

CONSTRUCTION OF LAMBDA TRP - TRANSDUCING

BACTERIOPHAGES IN VITRO

by

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A thesis presented for the Degree of
Doctor of Philosophy
of the
University of Edinburgh

Department of Molecular Biology
University of Edinburgh

December 1975



One thing I have learned in a long life:
That all our science, measured against
Reality, is primitive and childlike - and
Yet it is the most precious thing we have.

Albert Einstein

ACKNOWLEDGEMENTS

My thanks go to all the people who have made this work possible and who have contributed to making my stay in Edinburgh such a happy one. Apart from those too numerous to mention outside the Department of Molecular Biology, my thanks to those within the Department go to my supervisor, Dr Noreen Murray and my advisor, Dr William Brammar, for their constant guidance through these three years and for their tremendous patience and tolerance. I am grateful to Professor Martin Pollock for a place in this department and to the Medical Research Council for continuing financial support throughout my postgraduate studies. I am indebted to Drs Ken and Noreen Murray and Bill Brammar and to Sandra Bruce and Shirley Muir for constructing the phages in the first place.

Dr Ken Murray and his technicians Sandra Bruce and Karen Brown; Richard Thompson, Steve Hughes and Graham Brown have been a source of unlimited supplies of phage DNA's and enzymes, and Howard Cooke of the Department of Zoology and John Bishop of the Department of Epigenetics rescued us, and particularly me, when we exceeded the limits of these resources. I thank Dr Peter Highton for initiating me into the magic of electron microscopy and the world as seen through the eye of an electron. Thanks to Joey Rennie for the beautiful photographs and Shirley Muir for the more artistic of the diagrams; to Richard Lathe for the patent replicator and to my typist Lorna Dunford.

We are all in debt to the washing up ladies and the

ladies in the media room, Agnes, Beatrice, Ella, Frances, Kathy, Linda, Margaret, Marion and Winifred, who make life in the department so cushy, in spite of one, (who shall remain anonymous), who electrocuted me.

I wish to thank also Jean Beggs, Kate Borck, Anne Moir, Les Morss, John Atkins, Ricardo Pastrana, Algis Anilionis and numerous other members of the department for many hours of fascinating discussion. And last, but ~~most~~ of all, thanks to Susie, my constant companion at work these three years past, for her interest and unfailing support, moral and otherwise, not to mention technical help.

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Abstract

This thesis describes the construction in vitro of bacteriophage lambda-Escherichia coli tryptophan operon fusions by the sequential action of the restriction endonuclease R.HindIII and T4 polynucleotide ligase. Phages were isolated which complemented either the trpA, trpC or trpE genes. These phages carry three different fragments which are almost certainly adjacent in the trp operon of E. coli. The end points of the fragments carried by the phages have been mapped and shown to lie in the trpB and trpD genes. The orientation of the trp fragments within the receptor phage genome has been determined genetically and confirmed by heteroduplex mapping. The size of the fragments has been estimated from their mobilities on electrophoresis through agarose gels, and also by heteroduplex mapping.

Studies of the expression of the trp genes in these phages has shown that transcription initiated at the phage promoter P_L can continue through and beyond the phage attachment site, and that there is at least one constitutive promoter in the phage b₂ region to the left of R.EcoRI site 1 which initiates transcription rightwards.

Valuable information has been obtained concerning the merits of the receptor phage used in this study.

The centipede was happy, quite,
Until a toad in fun,
Said, 'Pray which leg goes after which?'
This worked his mind to such a pitch,
He lay distracted in a ditch
Considering how to run.

Anon

INTRODUCTION

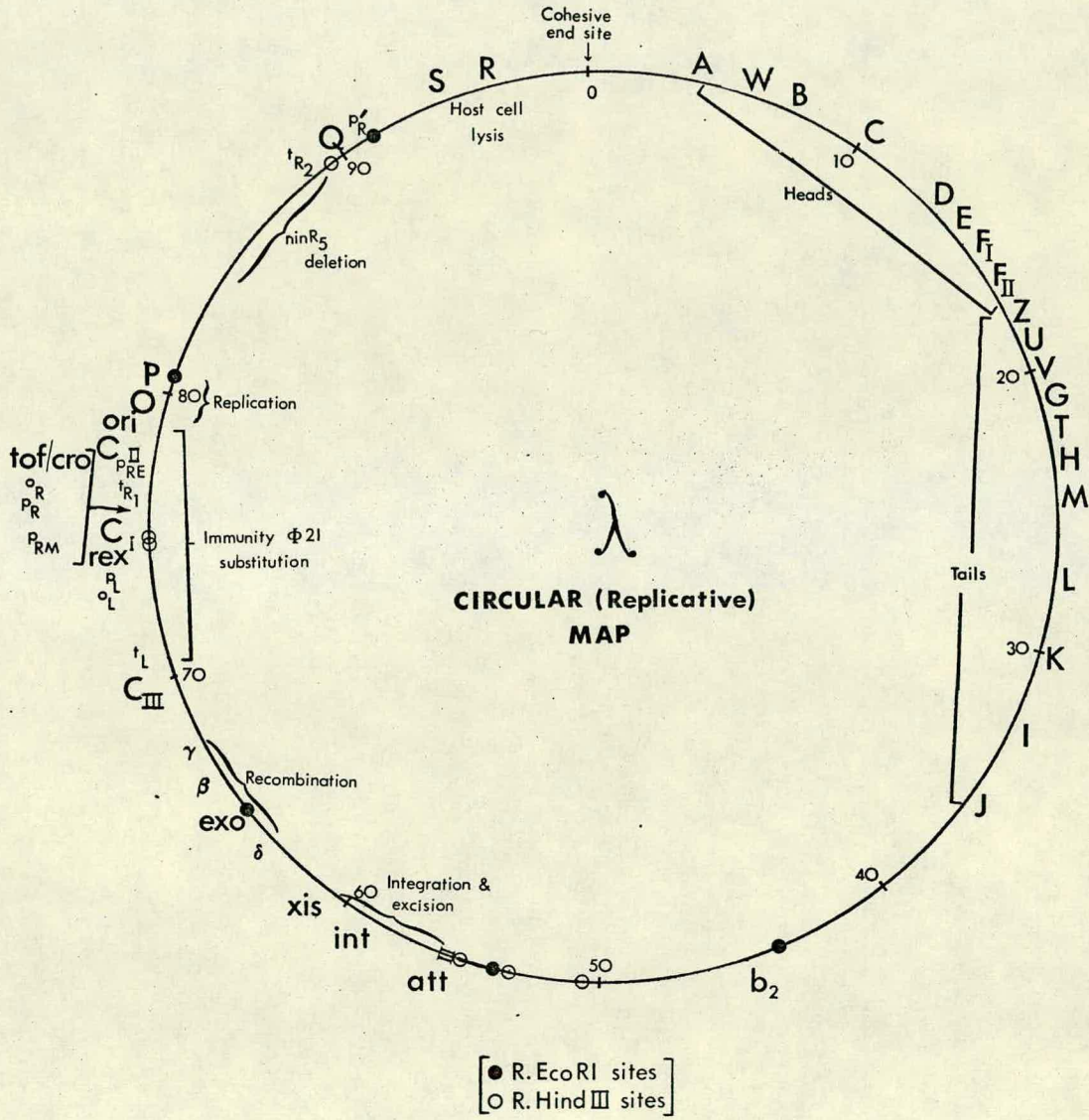
Lambda is a temperate bacteriophage which can follow two pathways upon infection of its host, Escherichia coli. It can develop lytically in the manner of a virulent phage and give a burst of eighty to one hundred progeny phage, or its DNA can undergo site-specific recombination to integrate into the bacterial chromosome (Gottesman & Weisberg, 1971). Here, with all genes repressed other than those in the same operon as the repressor protein, the phage is replicated as part of the bacterial chromosome and segregates with it at cell division (Sharp, Hsu & Davidson, 1972). In this state, termed lysogeny, the phage can exist indefinitely. Spontaneous induction to the lytic phase occurs at very low frequency, but treatments such as ultra violet irradiation, can induce the prophage into the lytic phase of development (Roberts & Roberts, 1974).

The stability of lysogeny depends upon repression of the phage genome (Sussman & Jacob, 1962), in particular, inhibition of its autonomous replication. This is achieved by the cI gene product, (lambda repressor), which binds to the left and right operators, (o_L and o_R), flanking it, thus preventing the expression of the genes dependent on the promoters p_L and p_R (Ptashne, 1971, and Fig 1).

The lysogenic response

Upon attachment of the bacteriophage tail fibres to the host cell wall, the linear phage DNA molecule is injected into the host and circularizes immediately due to the twelve nucleotide base, single-strand projections at each end of the vegetative DNA (Yarmolinsky, 1971). A DNA ligase mediates formation of 5'-3' phosphodiester linkages to convert this hydrogen bonded structure into a covalently closed circle (Gellert, 1967).

Fig 1



The phage DNA replicates bi-directionally (Schnös & Inman, 1970), until there are about twenty copies per cell (Young & Sinsheimer, 1968), replication requiring rightward transcription from p_R to supply the products of genes O and P (Joyner, Isaacs & Echols, 1966).

Transcription leftwards from p_L provides the int gene product which is necessary for integration of the phage DNA into the bacterial chromosome (Zissler, 1967). Once integrated, repression of the phage genome is established. The products of the cII and cIII genes act at a promoter, p_{RE} (promoter for repressor establishment), to initiate repressor mRNA synthesis (Echols & Green, 1971). This message is transcribed leftwards, reading through the cro gene backwards on the anti-sense strand, into the cI gene. Once sufficient repressor molecules (cI product) have accumulated in the cell further transcription of the lambda genome is blocked (Ptashne, 1971). cI gene expression then switches from p_{RE} to a more proximal promoter, left of p_R, p_{RM}, (promoter for repressor maintenance), (Echols & Green, 1971).

The Lytic Response

With wild type cells and phage, infection of a sensitive coli cell results in 30-50% of the phage forming stable lysogens; the others proceed into the lytic cycle. How the "decision" is made as to which pathway to follow is not understood. Herskowitz (1973), has discussed the possibility that the ratio of cI to cro proteins at a crucial time after infection may be the determining factor. There are host mutations which increase or decrease the frequency of lysogeny (Belfort & Wolff, 1971; Castellazzi, George & Butlin, 1972; Grodzicker, Arditti & Eisen, 1972). Kourilsky, Firshein & Gros (1975), have evidence that in a population of E. coli cells, those which are not replicating are

preferentially lysogenised. Study of the effect of the state of the host cell on the ability of the phage to lysogenise should help to explain what determines the fate of the infecting phage.

The lytic and lysogenic pathways are the same up to the point where integration would occur in lysogenic development and repression be established. In the lytic cycle the phage switches from bidirectional replication as a closed circle to replication as a rolling circle, fifteen to twenty minutes after infection (Skalka, Poonian & Bartl, 1972). The transcript initiated at p_R proceeds through the terminators t_{R1} and t_{R2}, under the influence of the N-gene product, into the late region of the genome. The product of gene Q is a positive regulator protein acting at a promoter just right of Q: p'_R (Herskowitz & Signer, 1970). In the absence of Q product, little late mRNA is synthesised and the levels of endolysin and structural proteins are very low (Hendrix, 1971).

The genes involved in head and tail synthesis are clustered on the genome. W to F_{II} are involved in head synthesis and assembly; Z to J in tails (Murialdo & Siminovitch, 1971). The A gene product cuts the DNA concatemers, resulting from rolling circle replication, at the cohesive end sites, (cos), (Wang & Kaiser, 1973). In addition, genes S and R, which are concerned with host cell lysis, are also in the late operon under Q control (Campbell & del Campillo-Campbell, 1963; Adhya, Sen & Mitra, 1971).

The Control Functions

The expression of the lambda genome in the lytic or lysogenic response is delicately controlled by the interplay of the products of genes N, cI and cro (Fig 2). The repressor (cI) and the cro product have an inhibitory effect on the transcription controlled by two

Legend to Fig. 2

The control functions within the immunity region of Lambda,
and the transcripts emanating therefrom

The central line indicates the immunity portion of the genome, extended to include the late terminator t_{R2} . The solid lines above and below represent the transcripts from this region. The arrowheads superimposed give direction, and an indication of the magnitude, of these transcripts. The dotted lines go from a control gene to its site(s) of action. A positive or negative sign alongside the line indicates respectively whether the product of the gene exerts a stimulatory or inhibitory effect on its target.

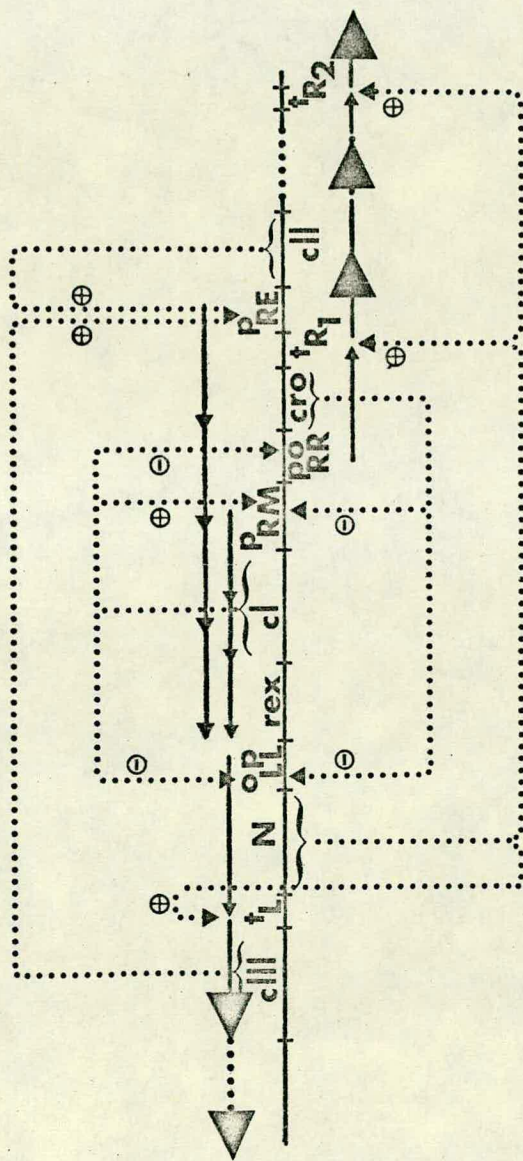


Fig 2

operators they interact with, o_L and o_R.

Cro

The cro product depresses the level of expression from o_L and o_R (Echols, Green, Oppenheim, Oppenheim & Honigman, 1973), but only quantitatively rather than absolutely (Franklin, 1971). This depression of transcription occurs at the stage in the lytic cycle when the phage switches from the early, bidirectional, mode of replication, to the late, rolling circle, mode. It probably effects the requirement to reduce synthesis of no longer needed early proteins, from the leftward reading N operon, and the cII, O and P products of the right operon.

Echols et al., (1973), have shown that the cro product probably binds to the same operator, o_L, as the cI protein, to repress leftward transcription, whereas for rightward transcription, the operator mutations v₁ and v₃, in o_R, which prevent binding of the cI repressor, do not substantially relieve the repression due to cro protein.

Cro does depress cII expression, so it must act at a site right of the cI gene. Thus the cro product indirectly inhibits repressor synthesis from p_{RE} by depressing the synthesis of cII and cIII proteins. In addition, cro product directly inhibits the transcription of the cI gene originating at p_{RM} (Reichardt, 1975).

cI

The repressor, the product of the cI gene, acts solely at the operators o_L and o_R (Ptashne, 1971). The sequence of these operators has been determined (Maniatis, Ptashne, Barrel & Donelson, 1974; Maniatis, Jeffrey & Kleid, 1975; Pirrotta, 1975). Both operators contain multiple repressor-binding sites of two-fold rotational symmetry,

separated by A/T-rich spacers. Both also contain an RNA polymerase-binding site similar to those found in the lac promoter and SV40 DNA. Mutations in both the operators are necessary for the virulent phenotype, where the phage proceeds inevitably into the lytic cycle, even upon infection of a cell lysogenic for lambda (Ptashne & Hopkins, 1968). The cI operon contains another gene, rex, which is expressed concomitantly with the cI gene. The only known function of the rex gene product is to exclude phage T4 rII mutants from developing in a lambda-lysogenic cell (Howard, 1967).

N

N-protein is a positive regulator, acting on the phage DNA at the termination sites t_L, t_{R1} and t_{R2}. In the absence of active N product a phage can only develop when it possesses a concomitant deletion of t_{R2} to allow late gene expression. There is a small amount of readthrough of t_{R1} to allow synthesis of enough O and P products for the phage to replicate (Heinemann & Spiegelman, 1970).

Adhya, Gottesman, and De Crombrughe (1974), and Franklin (1974) have shown that N protein interacts with RNA polymerase in such a way as to render it insensitive to a variety of termination signals; not only to the legitimate ones possessed by the phage, but also to those revealed by amber and ochre polar translation mutations. Adhya et al. and Franklin used the galactose and tryptophan operons, respectively, fused into the N operon. They have shown that the effect of N is seen only on transcription initiated at a phage promoter and that the suppression due to N is a suppression of polarity, and not of the mutation per se. The present model for N action is that it may prevent rho (Roberts 1969), from causing release of the prematurely terminated messenger, and either

immediately, or some distance downstream, allow RNA polymerase to continue transcription of the remaining genes in the operon.

Q

Q protein acts at the promoter p'_R to allow transcripts from p_R to read through into the late genes. In the absence of Q product there is a small amount of residual N-dependent transcription of the late genes (N dependent due to the requirement for anti-termination at t_{R2}). This transcription, as assayed by endolysin production, is only one fifteenth of that seen in the presence of Q (Dambly & Couturier, 1971).

The late genes of a prophage can be transactivated by Q product from a hetero-immune infecting phage. That is: Q protein can act in trans to stimulate transcription from p'_R in a fully repressed prophage, without inducing it (Szpirer & Brachet, 1970; Couturier & Dambly, 1970).

The "Non-Essential" Genes

Apart from the cIII gene, required for the initiation of repressor synthesis, the N operon, transcribed from p_L also contains at least five other genes whose products, although helpful, are not essential to lytic development of a phage.

That most proximal to N, following cIII, is gam, which directly inhibits the product of the host cell recB and recC genes, exonuclease V, (Unger, Echols & Clark, 1972; Unger & Clark, 1972). Enqvist and Skalka (1973), have demonstrated that gam protein is necessary to protect the product of rolling circle replication, linear DNA concatemers, from degradation by exoV. In the absence of gam product lambda can only mature its DNA by recombination between early, closed, circular, replicative intermediates (Skalka, 1975).

The phage possesses its own general recombination system, the

products of the red α and red β genes (also known as exo and β respectively). These proteins are the major influence on recombination between phage genomes. Because of the inhibitory effect of the gam product, the host recombination system makes only a minor contribution in most phage x phage crosses (Echols & Gingery, 1968; Signer & Weil, 1968a, 1968b). The product of the red α gene is known to be an exonuclease which degrades DNA from a 5' phosphate terminal residue in the 5' to 3' direction, either from a double stranded end, or from a gap in a one strand of a duplex, but it cannot degrade from a nick (a single break in the phosphodiester linkage between residues).

The function of β protein is not known. It has been partially purified and shown to increase the affinity of exonuclease for DNA (Radding & Carter, 1971). Skalka (1975) mentions the possibility that β protein may catalyze or stabilize the opening of a DNA duplex so that single stranded DNA could interact to form a synaptic recombination complex, a property similar to that postulated for the gene32 (Alberts) protein of T4 (Alberts & Frey, 1970).

Immediately to the right of the attachment site, are the genes int and xis. The products of these genes control the integration and excision of the phage DNA into, and from, the bacterial chromosome. Int product is required for the integration process (Zissler, 1967; Gingery & Echols, 1967; Gottesman & Yarmolinsky, 1968), while excision requires the products of both genes (Kaiser & Masuda, 1970; Guarneros & Echols, 1970).

The asymmetry of these processes has been used as an argument that the bacterial and phage attachment sites are not identical. Heteroduplexes between the two sites show no regions of homology (Davis & Parkinson, 1971).

The b_2 region of Lambda

This region between gene J and the attachment site is regarded as a non essential part of the lambda genome. Deletions of part or the whole region appear only to alter the buoyant density of the phage (although frequently such deletions originate at, and delete part of, the attachment site, thus making the phage integration defective). The evidence of Hendrix (1971), shows that, both early and late in the development of the phage, there are proteins which seem to arise as a result of transcription of this region, although no products of the b_2 region have any known function.

This evidence relies on a comparison of the proteins synthesised during infection with a wild type phage to those from infection with phages carrying deletions in this region. Hendrix detected seven proteins present early in wild type infection which are not present upon infection with b_2 deleted phage. At late times two additional proteins were detected. Excluding some function in the b_2 region which controls expression from another part of the lambda genome these proteins must be synthesised from message transcribed from b_2 .

Bøvre and Szybalski (1969), have shown that there is mRNA synthesised during lambda infection which is complementary to the b_2 region, as determined by hybridisation of the message to separated lambda DNA strands. They also found that there is overlapping right and leftward transcription, since some of the b_2 -specific message was self-complementary. There is recent evidence for repressor-independent promoters within the b_2 region (F. J. Blattner, pers. commun. via W. J. Brammar).

PROPHAGE INSERTION AND EXCISION

(Reproduced from Gottesman and Weisberg; 1971)

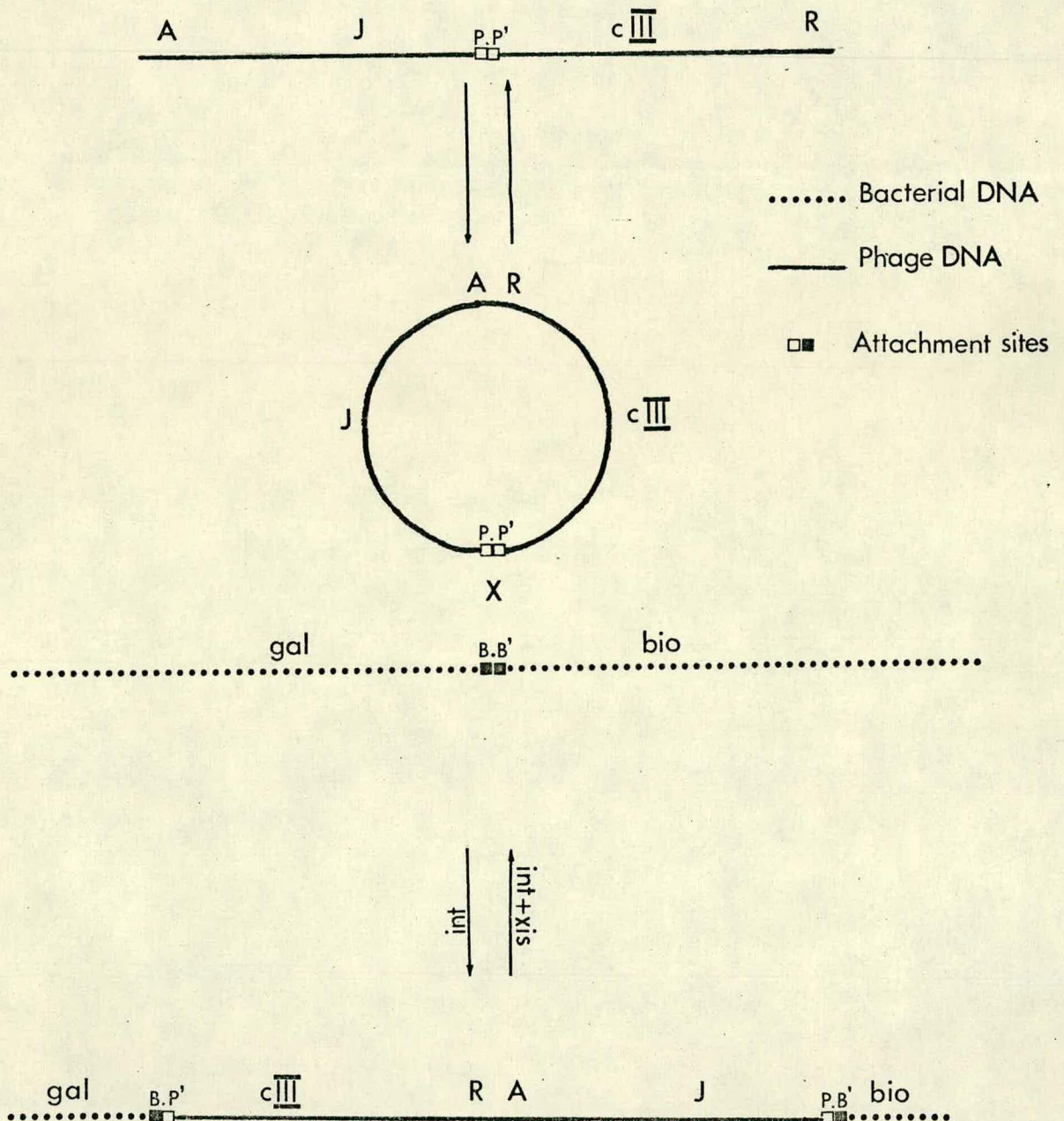


Fig3

The Generation of Transducing phages *in vivo*

Lambda (and related temperate coliphages 80, 82, 434 and 424), integrates into the E. coli chromosome by reciprocal, reversible, site-specific recombination. The model described by Campbell (1962), (Fig 3) is accepted as a satisfactory description of how this event might occur. Phage carrying portions of the bacterial chromosome, adjacent to the attachment site arise at very low frequency due to exceptional excision of the prophage by an "illegitimate" recombination event. These transducing phage carry variable portions of the bacterial DNA with concomitant loss of some phage DNA keeping the phage genome within the maximum size for packaging in the head.

For lambda, integrating between the gal and bio genes of the host, the transducing phage have bio DNA substituted into the N operon (Kayajanian & Campbell, 1966), or gal into the b₂ region (Signer, Manly, 1969), & Brunstetter, / (Fig 4). Lambda-bio phage are plaque-forming, unless the substitution extends into the N gene. Lambda-gal phage are frequently defective, the substitution often replacing some of the phage tail genes. Hybrid phage carrying the left arm and attachment site of Ø80 and the immunity region and control genes of phage lambda integrate at the attachment site for Ø80, between the trp operon and sup_F. Transducing phage arising by illegitimate excision of these phages carry portions of the trp operon substituted into the N operon. Such phage have mediated studies of expression from p_L and the mode of action of N gene product mentioned earlier (Franklin, 1971, 1974; Davison, Brammar & Brunel, 1974; Brammar, Murray & Winton, 1974).

Phage carrying portions of the bacterial genome other than that immediately adjacent to the attachment site can be constructed by forcing the phage to integrate at other places on the bacterial

ORIGIN OF TRANSDUCING PHAGES

(Reproduced from Gottesman and Weisberg :1971)

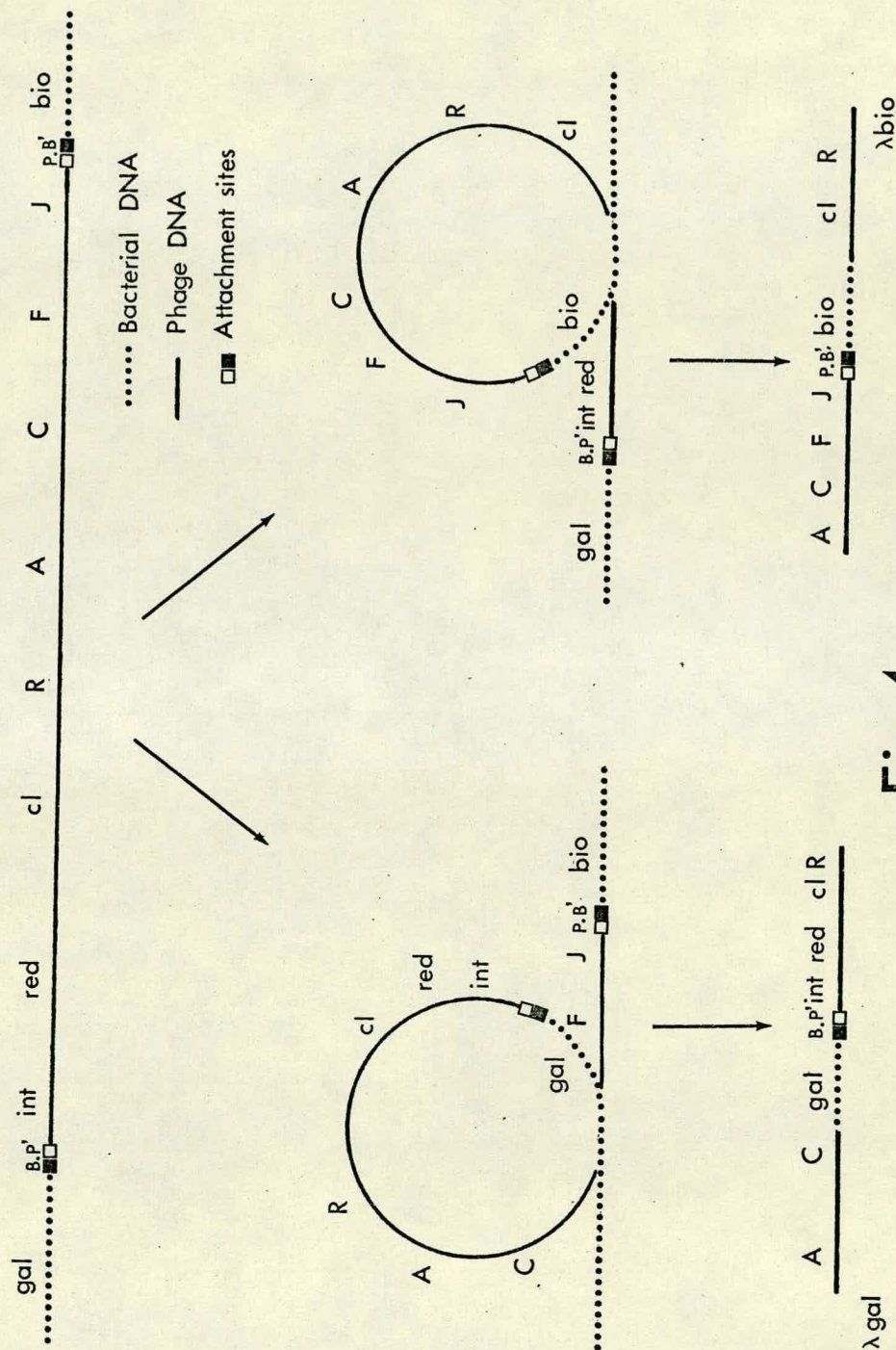


Fig. 4

chromosome when the normal attachment site is deleted (Shimada, Weisberg & Gottesman, 1972). Gottesman & Beckwith (1969) have described another method using transposition of genes from an F'factor, selecting for integration of these genes close to the attachment site.

Restriction Technology

Recently a much more versatile technique for isolating transducing phages and the manipulation of DNA in general, has been developed from the discovery that restriction endonucleases from various bacterial strains are able to cleave DNA, from virtually any source, at a defined set of sites.

Restriction endonucleases are responsible for the phenomenon of host-controlled restriction, manifested upon transfer of DNA between different strains of micro-organism. With bacteriophage lambda, the effect is seen as a reduction in the efficiency of plating (normally unity).

Phage grown on one strain are modified by the modification enzyme sharing site specificity with the restriction endonuclease. Modification usually consists of methylation of adenine or cytosine nucleotides within the restriction recognition sequence. The phage, if grown in a cell containing a restriction system of different specificity, will be restricted. The one in ten to fifty thousand phage which escape restriction will carry the host-specified modification, ensuring that any subsequent growth (in a cell of the new specificity) will be unrestricted. They will, however, lose the modification specified by the original system and thus be restricted if replated on that strain. (Reviews by Boyer, 1971; Arber, 1974).

There are two main types of restriction enzymes. Those classified as type I require S-adenosyl methionine (as donor of a methyl

group), ATP, and magnesium for their action; these include the coli A, K, and B systems. The type II enzymes require only magnesium and include the enzymes from Haemophilus influenzae dII and dIII, and that specified by the resistance transfer factor, RI.

The type I enzymes have specific targets in DNA which can be defined by mutations eliminating them (Murray, De Ritis & Foster, 1973; Murray & Brammar, 1973). Attempts to analyse the sequence cleaved by these enzymes have been consistently unsuccessful, probably because these enzymes do not cleave a unique sequence. The sequence which is modified may be an entry point for the endonuclease which, after binding, wanders along the DNA breaking it at a large number of sites (Horiuchi & Zinder, 1972; Brammar et al., 1974; Murray, Batten & Murray, 1973).

The type II enzymes do break the DNA at their respective recognition sequences and consequently produce a series of discrete and defined fragments which can be separated by agarose gel electrophoresis, velocity or equilibrium centrifugation (Allet, Jeppeson, Katagiri & Delius, 1973). Some of these type II enzymes leave single strand projections on the DNA which allow pieces from different sources to be subsequently annealed and sealed by the action of a polynucleotide ligase.

The principle of the method was first reported by Cohen, Chang, Boyer & Helling (1973), subsequent to the demonstration that the endonuclease RI (Yoshimori, 1971), generates cohesive ends from the sequence

$$\begin{array}{c} \downarrow \\ 5' - \text{GpApApTpTpC} - 3' \\ 3' - \text{CpTpTpApApG} - 3' \end{array}$$

$$\begin{array}{c} \uparrow \end{array}$$

(the arrows indicate the points of cleavage), (Hedgepeth, Goodman & Boyer, 1972). This sequence has two-fold rotational symmetry, meaning that any fragments with ends generated by R.EcoRI can be annealed in any orientation or order. The experiments of Cohen et al. (1973), used

plasmid replicons as the receptors for inserting R.EcoRI-created fragments. Fragments of Xenopus laevis ribosomal RNA cistrons have been inserted into a plasmid containing a single R.EcoRI target (Morrow, Cohen, Chang, Boyer, Goodman & Helling, 1974). These hybrid plasmids of eukaryotic and prokaryotic DNA replicate stably in E. coli, and RNA can be isolated which hybridizes to Xenopus rDNA. There is as yet no evidence that eukaryotic messenger coding for proteins can be translated in a prokaryotic cell, although a plasmid, carrying histone genes from sea urchin DNA, formed a polypeptide in a prokaryotic environment, but this was not identifiable as a histone (Kedes, Cohen, Houseman & Chang, 1975).

Murray and Murray (1974), have described the construction of lambda phages carrying single targets for the EcoRI endonuclease. Wild type lambda carries five targets for the EcoRI enzyme (Fig 1) and their positions have been determined (Allet et al., 1973; Thomas & Davis, 1975). Receptor phage are created using deletions covering sites, and by selecting for the loss of targets by enriching for resistant phage on passaging a phage between a restricting and non-restricting host for several cycles; (as described by Murray et al., 1973, for selecting derivatives of lambda resistant to the E. coli K restriction system).

The advantage of using lambda as a vector for heterologous DNA fragments is that a phage can be manipulated so as to select for insertions by creating a phage which is too small to be packaged unless it incorporates a fragment, such as replacement of the region between EcoRI sites 1 and 3 (Thomas, Cameron & Davis, 1974; Murray, Murray & Brammar, 1975). Combinations of deletions and immunity substitutions (i^{21} for i^λ) can be used to reduce the size of the phage

genome. Together with the ability of the phage head to tolerate a 10% excess over wild type genome length, this allows a heterologous DNA fragment 30% of the size of lambda to be incorporated (10^7 Daltons molecular weight).

In addition to EcoRI endonuclease the restriction enzyme HindIII (Roy & Smith, 1973) has been used as a tool for this work. This enzyme has six targets in lambda (Fig 1). These sites have two-fold rotational symmetry and are also cleaved to give complementary single stranded projections of four bases. These anneal more strongly than the EcoRI generated cohesive ends once they contain two G≡C pairs and two A=T pairs

5'-A↓pApGpCpTpT-3'
3'-TpTpCpGpApA-5' (the arrows indicate the points of cleavage)

Old, Murray & Roizes, 1975). Murray and Murray (1975), describe the construction of lambda receptor phage for DNA fragments created by this enzyme.

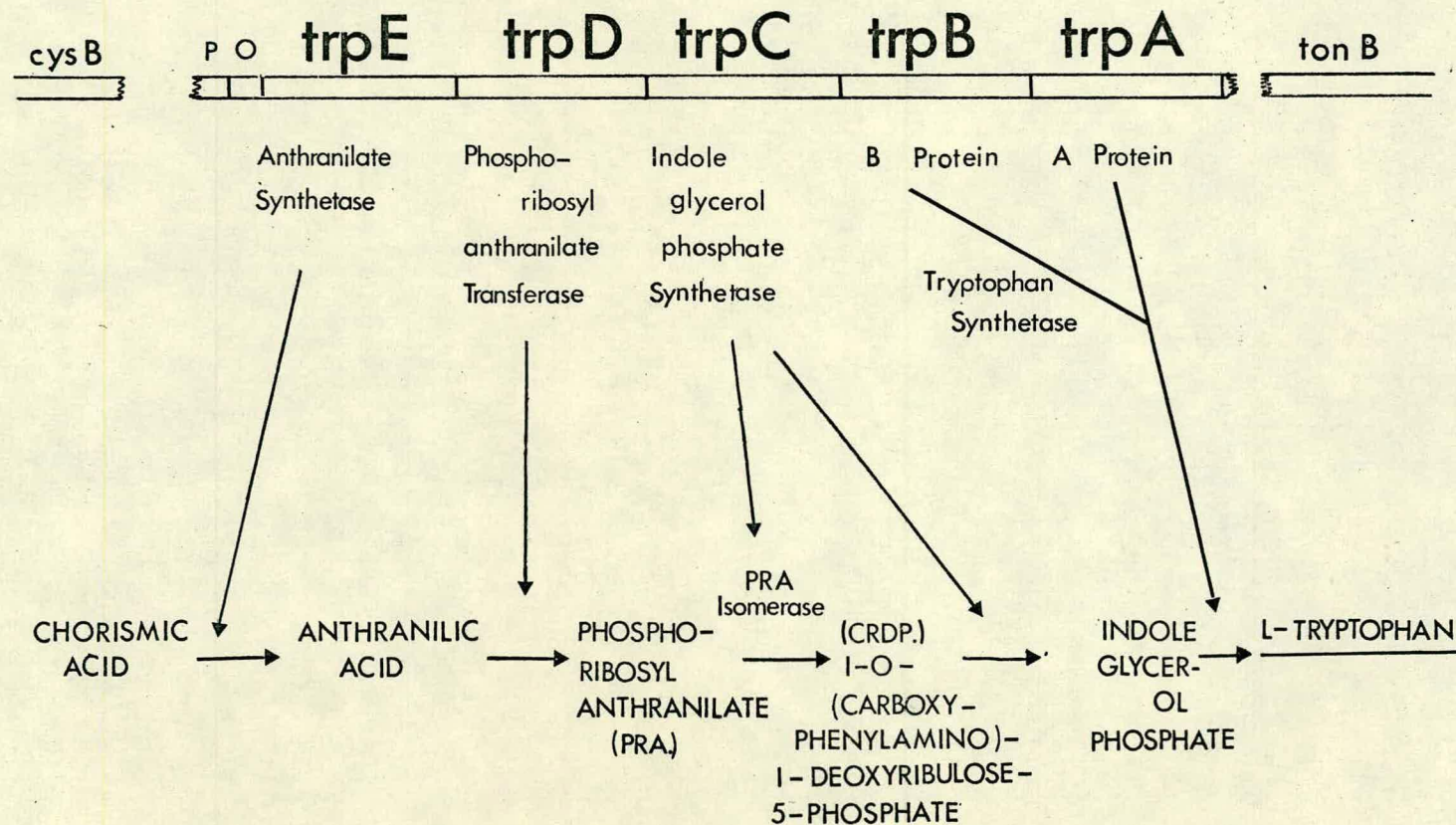
In addition to R.EcoRI and R.HindIII there are several other restriction enzymes for which lambda receptors can be constructed (Perricaud & Tiollais, 1975).

Lambda has the added advantage as a vector that it can, when integration proficient, be propagated as a lysogen. Lysis defective phage can be grown to a very high concentration in the host cell and then released by treating with chloroform. Thus large quantities of DNA can be isolated for sequence studies on inserted DNA, which can subsequently be cleaved from the phage DNA. Lambda is also useful for studies on the expression of inserted DNA, from its own promoter(s), or from the highly efficient promoter p_L on the phage. This allows the synthesis of large quantities of the gene products.

Using lambda trp-transducing phages constructed in vivo, Moir (1975)

Fig 5

THE TRYPTOPHAN OPERON OF E.COLI



showed that, expression from the trp-promoter combined with expression from p_L could result in the accumulation of 50% of the total cell protein as trp enzymes within one hour.

The Tryptophan Operon

The tryptophan operon of E. coli consists of five genes:- trpA, B, C, D and E, preceded by an operator and promoter (Fig 5). Biochemical assays exist for all five enzymes (Smith & Yanofsky, 1962). The trpE and D gene products comprise components I and II, respectively of the enzyme anthranilate synthetase. Component I alone will convert chorismate (the common precursor for the synthesis of aromatic amino acids), to anthranilate. It does this very inefficiently compared with its activity when complexed with component II (Ito & Yanofsky, 1966; Ito, Cox & Yanofsky, 1969).

The tryptophan synthetase enzyme, product of genes B and A, also acts as a complex, normally $\alpha_2\beta_2$, ($2\text{A} + 2\text{B}$ gene monomers). The β_2 subunit does not dissociate detectably under physiological conditions but it is a dimer of two identical polypeptide chains (Jackson & Yanofsky, 1969).

The five genes are transcribed as a single polycistronic mRNA from the trp promoter (Imamoto, Morikawa & Sato, 1965). Expression of the operon is repressed by high levels of tryptophan (Jacob & Monod, 1961) acting in concert with the product of the unlinked trpR gene (Cohen & Jacob, 1959). There is a low-level, constitutive promoter mapping at the promoter-distal end of the D-gene, p_{trpD} (Jackson & Yanofsky, 1972), giving residual expression, under repression, of genes C, B, and A to about 1% of the derepressed level.

Phages carrying genes of the trp operon fused to the N operon of lambda have proved valuable in studying the control of expression of

the lambda genome (Franklin, 1971, 1975; Davison et al., 1974; Murray & Brammar, 1973; Brammar et al., 1974). In this arrangement, the autonomous expression of the trp genes can be repressed by tryptophan and reading from p_L studied; under repression by cI product expression from p_{trp} can be followed.

In its own right, the trp operon, as reviewed by Bertrand, Korn, Lee, Platt, Squires, Squires & Yanofsky (1975), is unfolding a novel and important concept of the control of expression of a biosynthetic operon.

Certain internal deletions of the trp operon, removing a region preceding the first structural gene, E, but leaving the operator intact, show significantly increased expression of the remaining genes in the operon (Jackson & Yanofsky, 1973). The region deleted contained a leader sequence of some 160 nucleotides between the operator and the E gene (Bronson, Squires & Yanofsky, 1973; Bertrand, Squires & Yanofsky, in preparation, quoted in Bertrand et al. 1975). The repressor prevents transcription of the operon by inhibiting the binding of RNA polymerase molecules: polymerase molecules already transcribing the operon, or molecules reading into the operon from an upstream promoter, such as p_L in a trp-phage lambda fusion, are not affected by repressor (Squires, Lee & Yanofsky, 1975; Franklin, 1971). The deletions causing the increase in trp expression have a cis-dominant effect, thus they presumably remove an attenuator site or terminator, but this cannot be a site interacting with the trp repressor.

Studies of the levels of mRNA synthesised from p_{trp} showed a very high molar yield of message from the 160 nucleotide leader sequence compared with distal message from the structural genes of the operon.

The majority of transcripts terminate in the region of nucleotide 130 and were found to end in the sequence CU_8-OH or CU_7-OH , at the 3' terminus. (These sequences strongly resemble the termini of the early lambda 4S and 6S messages; U_6A-OH (Lebowitz, Weissman, & Radding, 1971; Rosenberg, Weissman & De Crombrughe, 1975)). In vitro this termination is not dependent on rho factor (Roberts, 1969).

Yanofsky and co-workers have recently isolated three new polarity suppressors (Karn & Yanofsky, in press, quoted in Bertrand et al., 1975), which do not insert amino acids in nonsense codons, but only restore the level of downstream message in a situation where the mutation would otherwise be polar. These suppressors also relieve the attenuation of the wild type trp operon in the derepressed state. The site of action of these suppressors is co-incident with the trp attenuator site.

Pouwels and van Rotterdam (1974) have isolated and partially purified an antitermination (At) factor from E. coli which stimulates synthesis of anthranilate synthetase in vitro. This factor specifically stimulates the transcription of trp messenger RNA.

The present hypothesis is that the attenuator represents a fine control mechanism regulating the expression of the trp-operon according to the growth rate of the cell. Starvation for tryptophan but no other amino acid relieves the attenuation (Bertrand et al. in preparation), so a response to cAMP or ppGpp levels through interaction with tryptophan-tRNA, tryptophanyl-tRNA synthetase or the At factor of Pouwels and van Rotterdam may be a likely possibility.

The similarity of this system, particularly of the polarity suppressors and At factor to the mode of action of lambda phage N protein on the terminators in the lambda genome is intriguing.

This thesis describes the construction, in vitro, of novel lambda transducing phages with the endonuclease R.HindIII, where phages with fragments of the tryptophan operon of E. coli were selected and studied. The tryptophan operon has been comprehensively analysed both in this laboratory and elsewhere, (see previous section). The existence of in vivo created trp-transducing phages for comparison and the availability of simple assays for the gene products has facilitated the characterisation of these in vitro trp-phages

The information obtained from this model system gives an insight into the problems which may be encountered in in vitro recombination experiments, and also into the advantages of isolating genes on transducing phages by in vitro methods. Techniques have been used which would be applicable to the characterisation of in vitro recombinants carrying other genes from E. coli and from other bacterial strains.

And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

T. S. Eliot

MATERIALS

Bacterial Strains

Strain	Relevant Characteristics	Reference	Source
W3350	<u>sup</u> ^o	Campbell (1961)	N. Murray, (N.M.)
Yme1	<u>supE</u> , <u>supF</u>	Bachmann (1972)	N.M.
C600	<u>tonA</u> <u>supE</u>	Appleyard (1954)	N.M.
QR47	<u>supE</u> <u>Rec</u> ⁺	Signer & Weil (1968a)	N.M.
QR48	<u>supE</u> <u>recA</u>	" "	N.M.
KB30trp2	(<u>att80 tonB trp</u>)delete	Denmey & Yanofsky (1974)	W. J. Brammar (W.J.B.)
C600(<u>imm</u> ^λ 21)			
W3110trpA ₈₈	<u>supF</u>	Yanofsky et al (1971)	W.J.B.
" <u>trpB</u> ₉₇₀₀		" "	I. P. Crawford
" <u>trpB</u> ₅₁		" "	"
" <u>trpB</u> ₃		" "	"
" <u>trpB</u> ₁₅		" "	"
" <u>trpB</u> ₆₂		" "	"
" <u>trpB</u> ₂₁		" "	"
" <u>trpB</u> ₅		" "	"
" <u>trpB</u> ₁₂		" "	"
" <u>trpB</u> ₂		" "	"
" <u>trpB</u> ₁₈		" "	"
" <u>trpB</u> ₄		" "	"
" <u>trpC</u> _{10243/9870}		" "	W.J.B.
" <u>trpD</u> ₁₅₉		" "	C. Yanofsky
" <u>trpD</u> ₅₆₂		" "	"
" <u>trpD</u> ₁₂₁₉		" "	"
" <u>trpD</u> ₈₇₅		" "	"
" <u>trpD</u> ₁₅₃₇		" "	"

W3110	<u>trpE</u> ₅₉₇₂	Yanofsky et al., (1971)	W.J.B.
"	<u>trpAC9</u> <u>tna</u> <u>tonB-trpABC</u> deleted	Franklin (1971)	W.J.B.
"	<u>trpAC9</u> <u>tna</u> <u>thy</u> ⁻	"	This thesis
"	<u>trpBE9</u>	Murray & Brammar (1973)	W.J.B.
"	<u>trpBE9</u> <u>thy</u> ⁻	"	W.J.B.
"	<u>trpAC9</u> <u>tna</u> (<u>imm</u> ^λ 21)		This thesis
"	<u>trpED102</u> <u>cys</u> ⁻ <u>trpED</u> deleted	Jackson & Yanofsky (1972)	W.J.B.
"	" <u>cys</u> ⁺	"	"
"	<u>polA</u> ₁	DeLucia & Cairns (1969)	N.M.
803	<u>supF</u> <u>rk</u> ⁻ <u>mk</u> ⁺	Wood (1966)	N.M.

Bacteriophage Strains

Strain	Reference	Source
λ^+	Kaiser (1955)	N.M.
λ_{cI}		N.M.
λ_{imm}^{21}	Liedke-Kulke & Kaiser (1967)	N.M.
540 see fig 1 in Results.	Murray & Murray (1975)	N.M.
$h^+ b_2^\nabla imm^{21} cI$		N.M.
$\phi 80pt190 \underline{trpA}_{96}$	Deeb et al., (1967)	W.J.B.
\underline{trpB}_{9578}		"
\underline{trpC}_{9941}		"
\underline{trpD}_{159}		"
\underline{trpE}_{9914}		"
$\phi 80^+$	Matsushiro (1963)	N.M.
426 see fig 1 in Results.	Murray & Murray (1974)	N.M.

Media

L-broth (Lennox 1955)

Difco tryptone 10g
Yeast extract 5g
NaCl 10g
+ H₂O to 1 litre

pH to 7.2 with NaOH

Vogel-Bonner salts (50x)

MgSO₄ 7H₂O 10g

CitricAcid H₂O 100g

(Vogel & Bonner 1956)

K₂HPO₄ 500g

Na(NH₄)HPO₄ 175g

+ H₂O to 1 litre

+ 2 mls chloroform as a preservative

Spizizen minimal salts (5x)

(NH₄)₂SO₄ 10g

K₂HPO₄ 70g

(Spizizen 1955)

KH₂PO₄ 30g

tri-sodium citrate 2H₂O 5g

MgSO₄ 7H₂O 1g

+ H₂O to 1 litre

Phage dilution buffer

KH₂PO₄ 3g

Na₂HPO₄ 7g

Glover (1962)

NaCl 4g

0.1M MgSO₄ 10 ml

0.01M CaCl₂ 10 ml

1% Gelatin (w/v) 1 ml

+ H₂O to 1 litre

<u>BBL bottom agar</u>	NaCl	5g
(Parkinson 1968)	BBL trypticase	10g
	Difco agar	10g
	+ H ₂ O to 1 litre	

<u>BBL top layer agar</u>	As above but only 6.5g agar
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<u>Water top layer agar</u>	New Zealand agar 6.5g
	+ H ₂ O to 1 litre

<u>L broth agar</u>	L-broth + 1.5% Difco agar
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<u>EMB agar</u>	Tryptone	10g
(Lederberg 1950)	Yeast extract	1g
	NaCl	5g
	Difco agar	15g
	Eosin	400mg
	Methylene blue	65mg
	K ₂ HPO ₄	2g
	+ H ₂ O to 1 litre	

Carbohydrate added to 0.1% w/v

P-medium

(Mandel and Higa 1974)

0.02M Phosphate pH7.0

0.015M $(\text{NH}_4)_2\text{SO}_4$

0.001M MgSO_4

1.8×10^{-6} M FeSO_4

1 mg/ml Glucose

0.1% Casamino acids

+ supplements for auxotrophic requirements

Vogel Bonner medium

10 ml 50x V.B. Salts

1.25 ml 20% Casamino acids

5 mls 20% Glucose

+H₂O to 500 ml

for inhibition of replication 5 mls of Uridine and 5 mls of
5-fluorouracil (from 2 mg/ml stock solutions) were added.

Tryptophan at 2 µg/ml was added for an infection experiment under non-
repressing conditions (lo trp), 200 µg/ml for repressing conditions
(hi trp)

Glucose minimal agar

15g New Zealand agar

H₂O to 800 mls

After autoclaving

200 mls 5 x Spizizen salts

10 mls 20% Glucose

ACH minimal agar

Glucose minimal agar

+ 0.04% Casamino acids

METHODS

Preparation of Plating cells (Murray, de Ritis & Foster, 1973)

Cells were grown in L-broth to 5×10^8 /ml, harvested, resuspended in 0.5x the original volume of 10^{-3} M MgSO_4 , and stored at 4°C .

Titration of phage (Murray et al., 1973)

0.1 ml of an appropriate dilution of phage in buffer was adsorbed to 0.2 ml of plating cells, at 37°C , for 15 min. 2.5 ml of top layer was added and poured immediately onto the plate. Incubation was for 12 hrs, or overnight, at the appropriate temperature.

Preparation of phage stocks by plate lysis (Murray et al., 1973)

A single plaque was picked using a sterile pasteur pipette and blown out into 1 ml of phage buffer containing a drop of chloroform and whirlymixed. 0.1 ml-0.2 ml of this was adsorbed to 0.2 ml of plating cells for 15 min. 1 ml of L-broth was added, followed by 2.5 ml top layer, and poured onto a freshly prepared L-plate. After incubation to confluent lysis (6-10 hrs), the top layer was harvested, treated with chloroform and spun at 2×10^4 g to remove agar and cell debris. The supernatant was collected and titred; titres ranged from 1×10^{10} - 5×10^{11} depending on the phage genotype.

Phage crosses (Murray et al., 1973)

0.2 ml of fresh plating cells were infected with a multiplicity of five of each parent phage. After 20 min adsorption, unadsorbed phage were removed by sedimentation of the cells and their subsequent resuspension in 1 ml of L-broth. 0.05 ml of this was then diluted into 10 ml of prewarmed L-broth and incubated for 2 hrs with vigorous aeration. The culture was then treated with chloroform, titred on a permissive host for total progeny, and on the selective host for the desired recombinant(s).

Construction of Lysogens (Jacob, Sussman, & Monod, 1962)

- i) For lysogenisation of a turbid phage by int-promoted site-specific integration between intact bacterial and phage attachment sites, 10^5 phage were spotted onto the centre of a bacterial lawn on an L-plate. After incubation, surviving cells were streaked from the centre of the spot. Single colonies from this were tested for the appropriate immunity.
- ii) For lysogenisation by rec-promoted integration between homologous bacterial DNA carried by the phage and the host. (The host carrying a deletion of the normal phage attachment site). A bacterial lawn was spotted with phage as before, after incubation cells from the centre of the spot were picked into L-broth, and 10^6 b₂ homo-immune clear phage of a different host range were added to challenge non-lysogenic or resistant cells. The culture was incubated overnight and surviving cells streaked and tested as above.

Test for integration proficiency (Gottesman & Yarmolinsky, 1968)

Putative lysogenic colonies were stabbed with a toothpick into an EMBO plate, seeded with 10^9 b_2 homo-immune clear phage of different host range. (For testing i^{21} lysogens only 10^7 phage per plate are used since i^{21} cI^- segregates virulent phage at high frequency; 10^{-8} per generation. (N. E. Murray pers. commun.)). Stable lysogens give healthy pink or red colonies; non-lysogens or segregants of abortive lysogens grow very poorly with dark mottled purple colonies.

Isolation of clear plaque derivatives of turbid phage (Kaiser, 1955)

10^5 phage were adsorbed to 0.2 ml of plating cells and plated on BBL agar. After overnight incubation at 37°C a few clear plaques could be discerned in the turbid lawn of confluent lysis. These were picked into 1 ml of phage buffer, diluted 10^{-4} - 10^{-5} , and replated for single plaques. After a second purification lysates were made from a single plaque.

Test for functional red α and red β products (Murray et al., 1973)

Serial dilutions of the phage were spotted onto a lawn of polA_1 cells. Red⁻ phage give tiny, if any, plaques, Red⁺ phage give normal size plaques.

Isolation of trp transducing phages (Franklin, 1971; Moir, 1975)

An appropriate dilution of the transducing lysate was adsorbed to 0.2 ml of plating cells, these cells carrying a particular auxotrophic lesion in the trp operon. After 15 min adsorption one drop of L-broth and 2.5 ml of Difco water top layer were added. This was poured onto an ACH plate and incubated 1-3 days. Transducing phage, complementing the host mutation, allow the cell to grow and lyse it, releasing tryptophan into the growth medium. This stimulates growth of the surrounding cells so the plaque appears with a ring of bacterial growth, or galaxy of colonies. These are commonly referred to as trp⁺ plaques.

Transduction by Lambda (Modification of Lennox, 1955)

10⁹ phage were adsorbed to 0.2 ml of plating cells. After 20 min the mixture was chilled, the cells and adsorbed phage pelleted and then resuspended in 1 ml 0.1M tri-sodium citrate. Samples of this were then spread undiluted, and at serial tenfold dilutions, on ACH plates. Transductant colonies appeared after 1-3 days incubation.

Hfr conjugation (Lederberg & Tatum, 1946; Miller, 1972)

Cells were grown to 2×10^8 /ml in L-broth. Male and female cells were mixed in equal proportions and incubated for the desired time at 37°C. Mating pairs were disrupted using a whirlymixer and 0.2 mls spread immediately undiluted and at serial 10-fold dilutions, on selective medium. Ex-conjugants were repurified twice by streaking for single colonies on selective medium.

Trimethoprim selection for thy⁻ cells (Modification of Stacey and

Simson, 1965, by Miller, 1972)

Cells from a fresh overnight culture were washed with 10^{-3} M MgSO_4 . 0.2 ml was spread onto a minimal-glucose plate containing 200 $\mu\text{g/ml}$ thymine and 10 $\mu\text{g/ml}$ trimethoprim. The small colonies growing after 2-3 days incubation at 37°C were repurified on the same selective plates. They were then tested for their inability to grow in the absence of thymine.

Ultra-violet induction (Miller, 1972)

i) To make a phage lysate:- Lysogenic cells were grown to $2 \times 10^8/\text{ml}$ in L broth, harvested and resuspended in half their original volume of 10^{-2} M MgSO_4 . After irradiation with 400 ergs/mm^2 they were diluted 4x into pre-warmed L-broth and incubated for 2 hrs at 37°C , in the dark, with vigorous aeration. The lysate was treated with chloroform and cell debris removed by low-speed centrifugation.

ii) For the screening of the comparative inducibility of lysogens.

A petri-dish was prepared with drops of liquid, on a grid of 50 squares (Miller, 1972), such that 30 μl of a lysogen in L broth at 2×10^7 cells/ml was in the first drop, followed by four drops of phage buffer, each of 30 μl . Then 30 μl of another lysogen in L broth, 4x30 μl drops of buffer, and so on. The plate was incubated at 37°C for three hours, irradiated with U.V. at 400 ergs/mm^2 , covered with tinfoil, and replaced at 37°C for a further two hours. Serial dilutions of each lysogen were then made using microcap pipettes, (5 μl into 30 μl from one drop to

the next). These drops were then replicated onto an L-plate for a count of the surviving cells, (using a device with 50 pins, kindly donated by R. Lathe, similar to that described by Szybalski, 1956). Several drops of chloroform were then put on the original plate. This was left for 20 mins to kill the remaining cells. The drops were then replicated again, this time onto a plate previously overlaid with a lawn of sensitive cells in 1% BBL top layer agar, to assay phage production.

Preparation of phage by liquid lysates (Thomas & Abelson, 1966)

Cells were grown in L broth plus 10^{-2} M MgSO_4 to 2×10^8 /ml. Phage were added at a multiplicity of one, and the incubation continued, with vigorous aeration, until the optical density at 670 nm had reached a minimum. Chloroform was added (5 mls/litre) the lysate shaken and left to stand for 20 min; then the cell debris was pelleted by centrifugation at 2×10^4 g for 10 min at 4°C .

Concentration of phage by low and high speed centrifugation (as above)

Phage were pelleted at 4×10^4 for 2 hrs at 4°C . The phage were resuspended by gentle shaking overnight in 1/20 the original volume of phage buffer. The resuspended phage were spun at 2×10^4 g for 10 min, the supernatant collected, the pellet re-extracted with 1-2 ml phage buffer, spun as before, and the supernatant added to that from the first extraction. DNase and RNase were added to a final concentration

of 10 $\mu\text{g/ml}$ each and the lysate digested for 1 hr at room temperature. The phage were pelleted again by high speed centrifugation, and the pellets resuspended, recentrifuged at low speed, and re-extracted as before. Caesium chloride was added to 41.5% w/w and the suspension clarified by spinning at $2 \times 10^4 \text{ g}$ for one hour. The suspension was decanted into clean tubes and centrifuged to equilibrium for 24 hr at $1 \times 10^5 \text{ g}$ at 4°C . The phage were collected from above using a 19 gauge needle, and re-banded in pre-clarified 41.5% CsCl.

Phenol extraction of DNA (Thomas & Abelson, 1966)

The phage were dialysed against 10^{-2} M Tris-HCl; 10^{-3} M EDTA, pH 8.0, for one hour to remove the caesium chloride. The DNA was then extracted four times with equal volumes of freshly distilled phenol, pre-equilibrated with 0.5 M Tris pH8.0. The phenol was removed by exhaustive dialysis against four changes of 10^{-2} M Tris; 10^{-3} M EDTA, over 24 hrs. The O.D. at 260nm and 280nm was measured for a ratio of 1.8 or better. Phage escaping extraction were assayed by transfection at a DNA concentration of 1 $\mu\text{g/ml}$.

Restriction of DNA with EcoR_I and Hin_{dIII} endonucleases (Murray &

Murray, 1974; Allet & Solem, 1974; Allet, Jepeson, Katagiri & Delius, 1973)

DNA was restricted in Tris-HCl, MgCl_2 , and β -mercaptoethanol, all to a final concentration of 10mM. For EcoR_I, NaCl at 100mM was added to

inhibit EcoR_I' . For Hind_{III} , NaCl was added to 50mM. Incubations were continued for 30-60 min; usually 1-5 μl of enzyme were used per microgramme of DNA. For fragment separation on agarose gels, the reaction was stopped with excess EDTA (10mM); for subsequent ligation the reaction was stopped by heating to 70°C for 10 min.

Agarose Gel Electrophoresis (Murray & Murray, 1974; Hayward & Smith, 1972; Allet *et al.*, 1973; Thomson, Hughes & Broda, 1974)

Gels were 1% agarose in 0.04M Tris, 0.02M sodium acetate, 0.01M EDTA, pH to 8.2 with acetic acid (buffer E). They were cast between 40 cm x 20 cm glass plates, 0.3 cm apart, with 1 cm wide wells, made with a perspex slot former. Samples were loaded in a solution of 50% glycerol, 50% buffer E, with 0.4% Bromophenol blue; they were desiccated under vacuum to a volume of 10 μl before loading. Gels were run at 40mA, 200V, for 12-15 hr, then stained with a solution of ethidium bromide (EtBr in buffer E), at a concentration of 0.5 $\mu\text{g/ml}$. The fluorescence of the DNA bound EtBr under ultra-violet irradiation was photographed, through a 4x red filter, on Ilford FP4 film. (ChromatoVue, UV Products Inc., San Gabriel, Cal, USA).

T4-ligase joining of DNA fragments (Richardson, Masamune, Lire, Jaquemin-Sablon, Weiss & Fareed, 1968; Nath & Hurwitz, 1974; Murray & Murray, 1974)

DNA fragments were reacted with T4-ligase in 1/10 dilution of the following cocktail, 0.66M Tris, pH 7.5; 0.01M EDTA, pH 9.0; 0.1M $MgCl_2$; 1 $\mu g/ml$ BSA; 0.1M di-thiothreitol; 0.01M ATP. After the heat pulse following restriction the DNA was iced, then water added to give a final DNA concentration of 16-20 $\mu g/ml$, (plus NaCl to 0.1M allowing for the subsequent addition of the reaction cocktail). This mix was incubated for 10 min at 37°C, iced, then the cocktail added, with ligase to 2 $\mu l/ml$. (Ligase activity: 100 units/ml, 428 units/mg protein; One unit converts one nmole of ^{32}P -phosphomonoesters into a phosphatase resistant form in 20 min at 37°C.)

The reaction was incubated at 10°C for 5 hr, then 2-10 days at 0°C. Samples were assayed for activity by transfection at intervals over this period.

Transfection. (Mandel & Higa, 1970; A. E. Jacob & S. J. Hobbs, personal communication to N. E. Murray)

Cells from a fresh culture in L-broth were diluted to 2×10^7 in P medium and incubated overnight. These were diluted 1/20 into fresh P medium and grown to $10^9/ml$. The cells were chilled, harvested by centrifugation, resuspended in half the original volume of cold 0.1M $CaCl_2$ and left on ice for 20 min. They were then harvested again and resuspended in 1/10 the original volume of 0.1M $CaCl_2$. They were left at least 15 min on ice before use for transfection.

0.1 ml of DNA at a concentration of 1 $\mu\text{g}/\text{ml}$ in 1x SSC, (0.15M NaCl; 0.015M tri-sodium citrate, pH 7.0), were added to 0.2 ml of competent cells. The mixture was heat pulsed at 37°C for 30 sec, then left on ice, with occasional shaking, for at least 90 min. Samples of the mix were then plated undiluted, and at serial 5x dilutions, with 2.5 ml of BBL top layer, supplemented with 10^{-3}M MgSO_4 . Plaques were sometimes picked individually, but more usually they were harvested in the same way as a plate lysate.

Infection Experiment (Franklin, 1971; Moir, 1975)

Cells were grown in L-broth to $5 \times 10^8/\text{ml}$, (supplemented with 100 $\mu\text{g}/\text{ml}$ thymine if Thy^-). The culture was chilled, harvested by centrifugation at 4°C, and resuspended in half the original volume of cold 10^{-3}M MgSO_4 , 20 $\mu\text{g}/\text{ml}$ 5-fluorouracil. The cells were centrifuged again and resuspended in 1/10 the original volume of the same solution. The cells were infected with the test phage at a multiplicity of two, left to adsorb for 20 min at 37°C, chilled on ice, and harvested again. The cells were resuspended to $5 \times 10^8/\text{ml}$ in pre-warmed Vogel-Bonner medium, and incubated with vigorous aeration. The supernatant was titred for unadsorbed phage. Samples of 20 ml were taken at intervals and iced immediately, the time of resuspension being taken as zero. The samples were centrifuged at $1 \times 10^4 \text{ g}$ for 10 min, the cells were washed with cold 0.1M Tris-HCl, pH 8.0, harvested and resuspended in sonication buffer, (0.05M Tris-HCl; 10^{-3}M EDTA; 10^{-3}M β -mercaptoethanol; pH 7.8).

The cell suspensions were sonicated for 6 sec in ice, left 30 sec, and sonicated again for 6 sec, on an MSE 100W Ultrasonicator. Cell debris was removed by centrifugation and the supernatant assayed for enzyme activity and protein content.

Anthranilate synthetase assay (Smith & Yanofsky, 1962)

Anthranilate synthetase activity was assayed in a freshly prepared mixture of substrates containing $2 \times 10^{-4} \text{ M}$ chorismic acid, (Gibson, 1964); 10^{-3} M β -mercaptoethanol; 10^{-2} M glutamine; 10^{-2} M phosphate buffer, pH 7.0; $4 \times 10^{-3} \text{ M}$ MgSO_4 . 0.1 ml of enzyme sample was mixed with 1.5 ml of assay mix pre-warmed to 37°C . The conversion of chorismate to anthranilate was observed using a Locarte recording spectrofluorimeter with a constant temperature cuvette holder at 37°C . The increase in fluorescence at 386 nm was followed using an excitation wavelength of 313 nm. The recorder was calibrated with 10^{-7} M anthranilate. Enzyme activity was expressed as units per mg of protein where one unit is the amount of enzyme producing 10^{-7} moles of anthranilate in 20 min at 37°C . (To assay component I, alone, of the anthranilate synthetase complex, (the E-gene product,) in the absence of component II, (the D-gene product), 0.04 M $(\text{NH}_4)_2\text{SO}_4$ was substituted for glutamine in the assay mix).

Tryptophan Synthetase Assay (Smith & Yanofsky, 1962)

Tryptophan synthetase activity was measured in a mixture comprising 3.0 ml DL-serine, 21 mg/ml; 1 ml pyridoxal 5-phosphate, 0.1 mg/ml; 0.3 ml saturated NaCl; 1.6 ml indole, 0.295 mg/ml; 2.1 ml water. 0.2 ml of enzyme sample plus 0.8 ml of substrate mix were incubated together for one hour at 37°C. 0.1 ml of 1M NaOH was added to stop the reaction. Samples were extracted with 4 ml of toluene. After separation 1 ml of the toluene layer was mixed with 4 ml of ethanol plus 2 ml of indole reagent (5 parts of 5% para-dimethyl amino benzaldehyde in 95% ethanol to 12 parts of 8% H₂SO₄ in ethanol).

After allowing the colour to develop for twenty minutes the optical density at 540nm was read against a blank of toluene extracted water. Enzyme activity was expressed in Units/mg of protein, where 1 Unit is that amount of enzyme converting 10⁻⁷ moles of indole to tryptophan in 20 min at 37°C.

Protein Assay - (Lowry, Rosebrough, Farr & Randall, 1951; Layne, 1957)

0.05 ml of extract was mixed with 1 ml of water and 5 ml of Folin's reagent. This was left to stand for 10 min at room temperature, (20-24°C). 0.5 ml of Folin Ciocalteu's reagent, (freshly diluted 1:1 with distilled water), was added, mixed immediately, and left to stand for 20 min. The O.D. at 670nm was recorded against a blank without protein. A calibration curve was plotted from standard concentrations of BSA solution.

& Davidson, 1971).

5×10^{10} particles of each phage were denatured in 0.5 ml 10^{-1} M NaOH; 20 mM EDTA, for 10 min at room temperature. This solution was neutralised with 50 μ l 20mM Tris. 1.575M HCL: 0.5 ml 99% Formamide, and left for 2 hr to renature. This was then dialysed overnight against 10^{-2} M Tris; 10^{-3} M EDTA at 4° C. 50 μ l of the renatured DNA solution was then diluted into 1 ml of hyperphase, (10^{-1} M Tris: 10^{-2} M EDTA; 50% Formamide: pH 8.5), and left for 2-3 min. 50 μ l of cytochrome C solution was added, (1 μ g/ml in 2×10^{-2} M Tris. 2×10^{-3} M EDTA; pH 8.5). 50 μ l of this solution was spread onto 100 ml hypophase, (10^{-2} M Tris; 10^{-3} M EDTA; 15% Formamide; pH 8.5), by slowly running the hypophase down a fused quartz ramp, entering the hypophase at an angle of 30° to horizontal. The hypophase was contained in teflon dish with a 10 cm x 10 cm well. After allowing the film to spread for 2 min, the DNA was picked up on freshly prepared, collodion-coated, copper grids. These were stained for 30 sec in Uranyl acetate, (5×10^{-2} M Uranyl acetate; 5×10^{-2} M HCl; in 90% ethanol), and washed for 10 sec in 90% ethanol. All solutions were made up immediately before use. Spreading was carried out in an air-tight room under a hood, to minimise the effects of draughts. Air movements were monitored using smoke, and movements of the film observed by a tiny quantity of talc placed on the hypophase before spreading commenced. Only films which spread in conditions of still air, and where the talc moved at least 5 cm away from the ramp were picked up on grids.

The grids were rotary shadowed with platinum at a distance of 4 cm from, and 0.5 cm below the platinum. Some grids were also stationary

shadowed, at the same distance away from the platinum, but only 0.25 cm below it, (Thomson, Escaronis, Parker, Slater, Doniger, Tessman & Werner, 1975). The grids were then coated with vaporised carbon and the collodion subsequently removed with absolute alcohol.

The grids were observed in a Siemens Elmiskop 1A electron microscope, at an accelerating voltage of 80KV, and 4×10^4 x magnification. Photographs were taken at 2×10^4 x magnification on glass photographic plates. A five times enlargement of the plates was used to draw contours which were measured using a map-measurer.

Lambda, lambda, burning bright,
In the test tube, our delight,
Who can say what will be told
Of our endeavours, brave and bold.

We have ligased bits and pieces,
DNA's of varied species,
Cut from fruit flies, ducks and weasels,
Next, the virus causing measles.

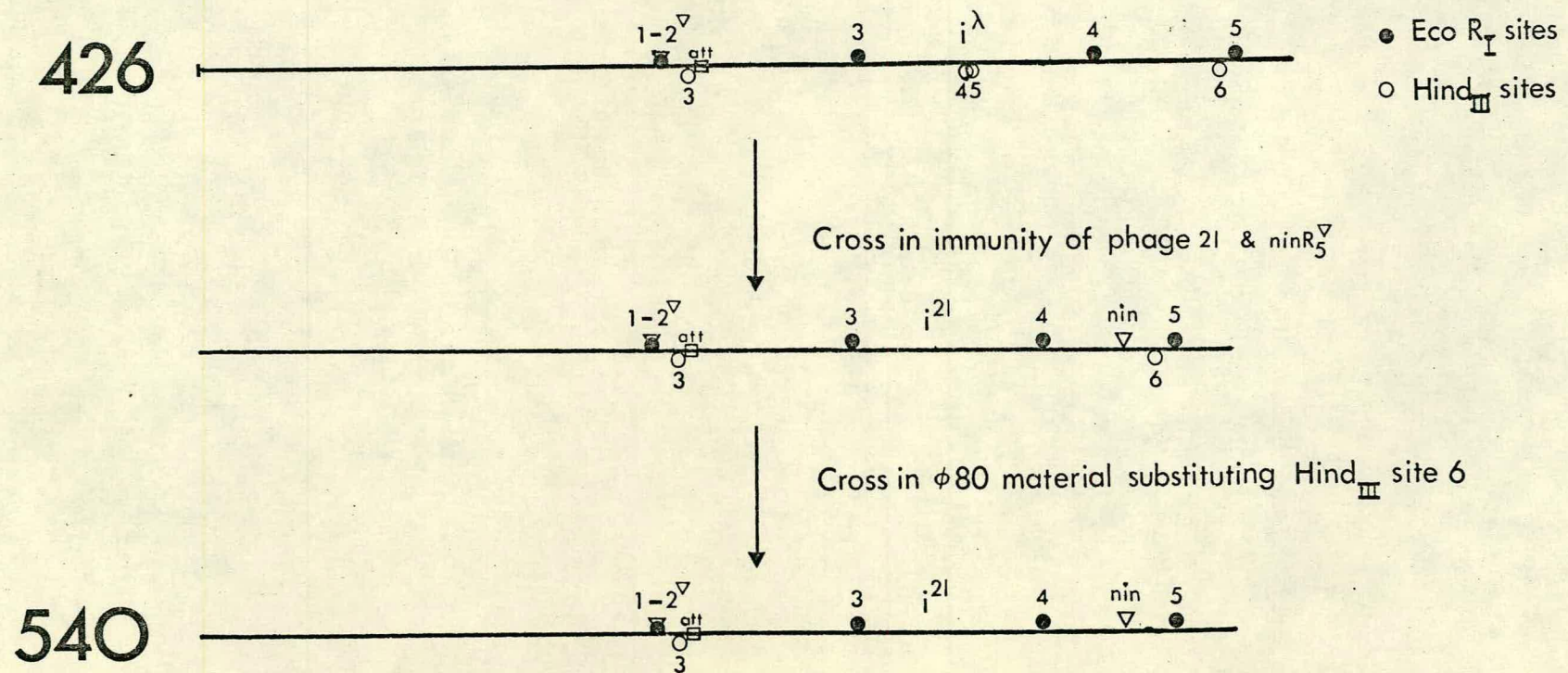
The pressmen wait with bated breath,
For us to cause a plague of death,
Believing that in our white tower,
We mad boffins seek world power.

(With apologies to William Blake)

Fig 1

ORIGIN OF 540 RECEPTOR PHAGE

a derivative of bacteriophage lambda.



RESULTS

Construction of the Phages

This thesis describes the characterization of a group of plaque-forming trp-transducing phages, constructed in vitro. These phage are the result of restricting the receptor phage 540 with the R.HindIII restriction endonuclease, and incubating it in the presence of polynucleotide ligase with fragments from E. coli W3110 DNA digested with the same enzyme. Fig 1 shows the structure and part of the derivation of phage 540, (Murray & Murray, 1975).

Subsequent to ligation the reconstituted phage DNA molecules were recovered as plaques by transfection of strain 803 supF trp[∇]. The transfected bacteria were plated on BBL agar containing 10^{-2} M MgSO₄, and incubated overnight. The five to six hundred plaques which appeared on each plate were harvested by collecting the whole top layer and treatment as if it were a phage lysate. A total of 28 lysates were collected by this means.

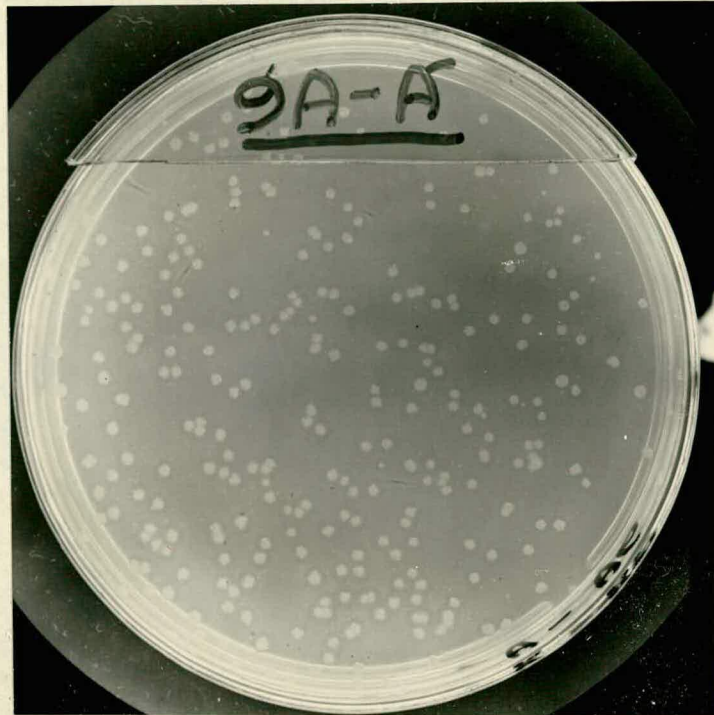
Isolation of the phages

The harvests of transfectants were screened for their ability to complement different E. coli strains each with an auxotrophic lesion in one of the five trp genes, (Franklin, 1971; Moir, 1975). Here the cell is provided with the missing enzyme enabling it to grow on a medium lacking tryptophan. When the phage lyses the host tryptophan is released stimulating the growth of adjacent bacteria. This leads to the formation of the characteristic "trp⁺ plaque", an area of growth around a plaque, the centre of which will contain stable trp⁺ lysogens if the phage is a turbid plaque former. Plates 1-14 are photographs of such plaques; plate 15 is an enlargement of one, showing the structure in detail.

Legend facing plates 1-15

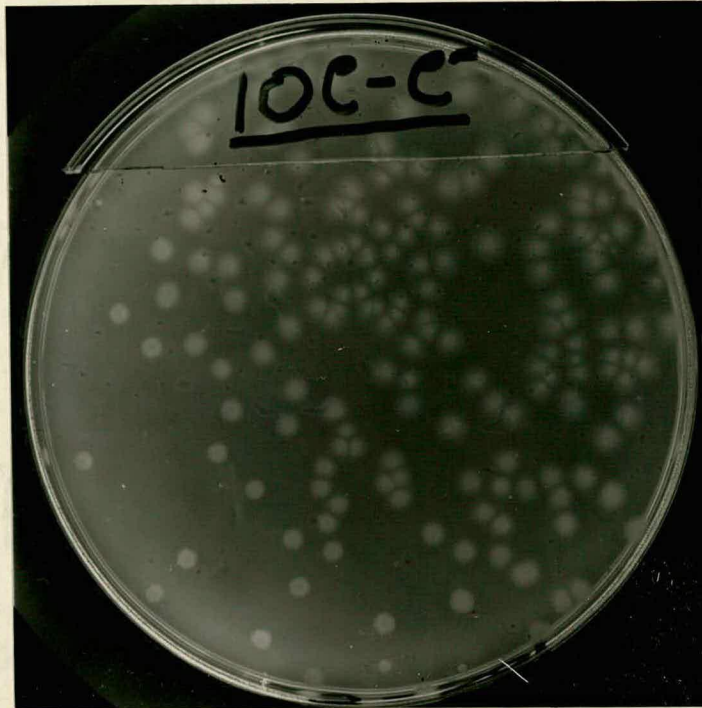
Plates 1-15 show trp⁺ plaques. The label on the plate shows the phage giving the plaques, (its isolate number followed by the trp gene it carries), and after the hyphen the trp mutant strain it is plated on. A⁻ is W3110 trpA₈₈; B⁻ is W3110 trpB₉₇₀₀; C⁻ is W3110 trpC₁₀₂₄₃/C₉₈₇₀; D⁻ is W3110 trpD₁₅₉; E⁻ is W3110 trpE₅₉₇₂. Phages 19A and 19C carry trp A, B and C and complement strain W3110 trpAC9 which is deleted from tonB through A, B, and most of trpC (fig 5)

1

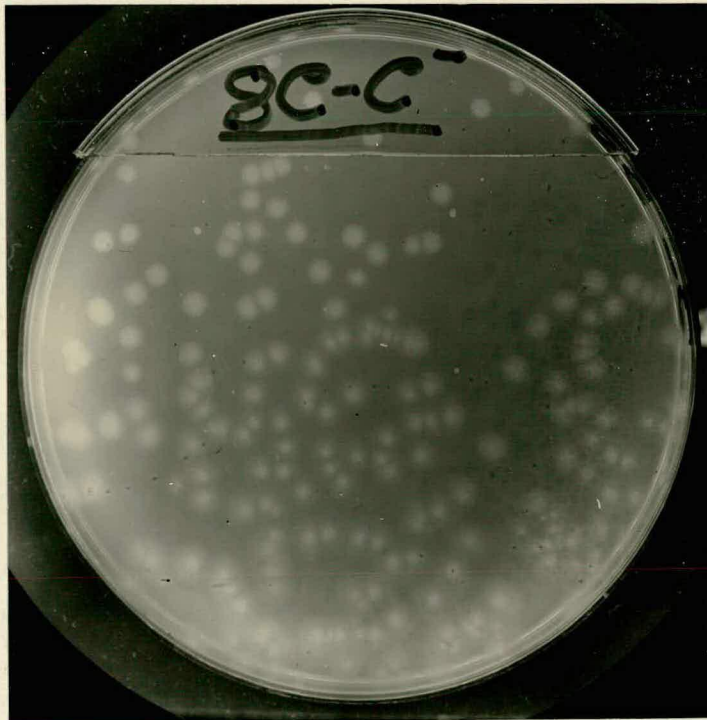


2

3



8C-C



4

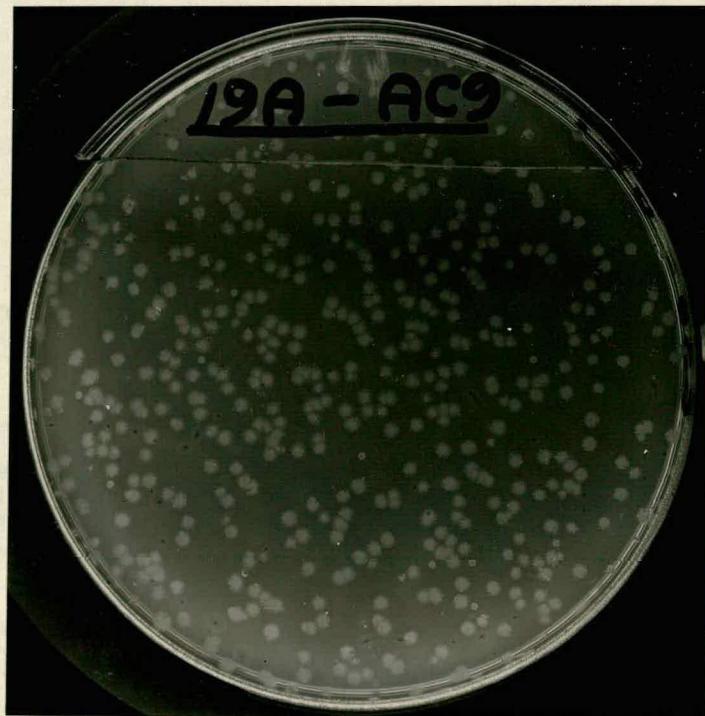
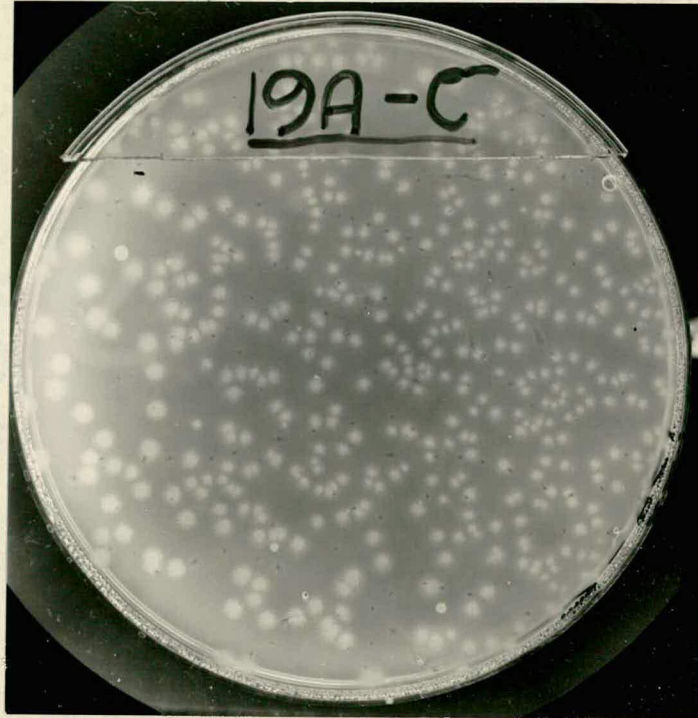


5



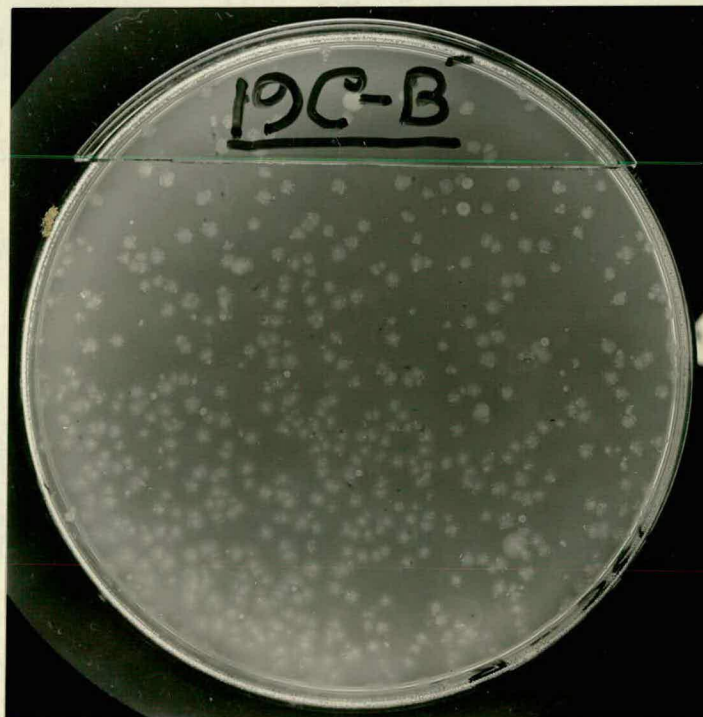
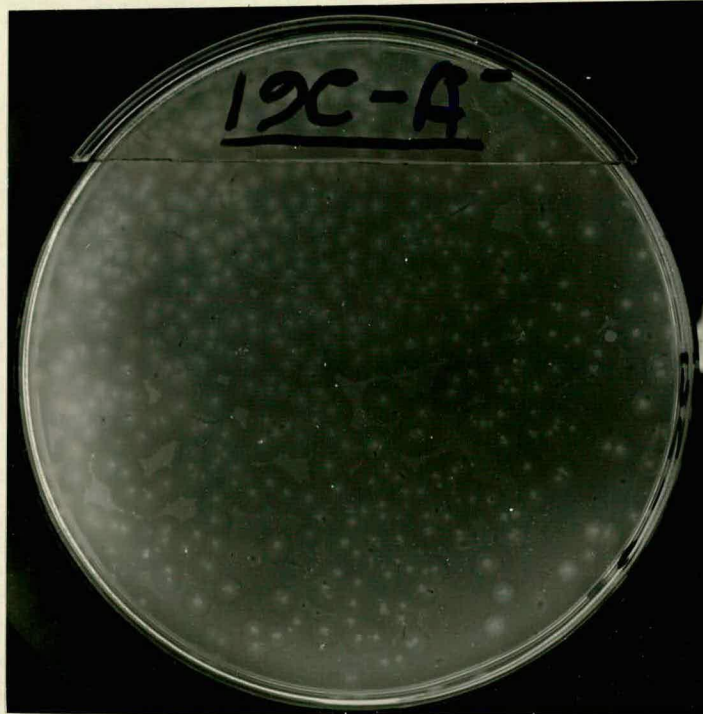
6

7



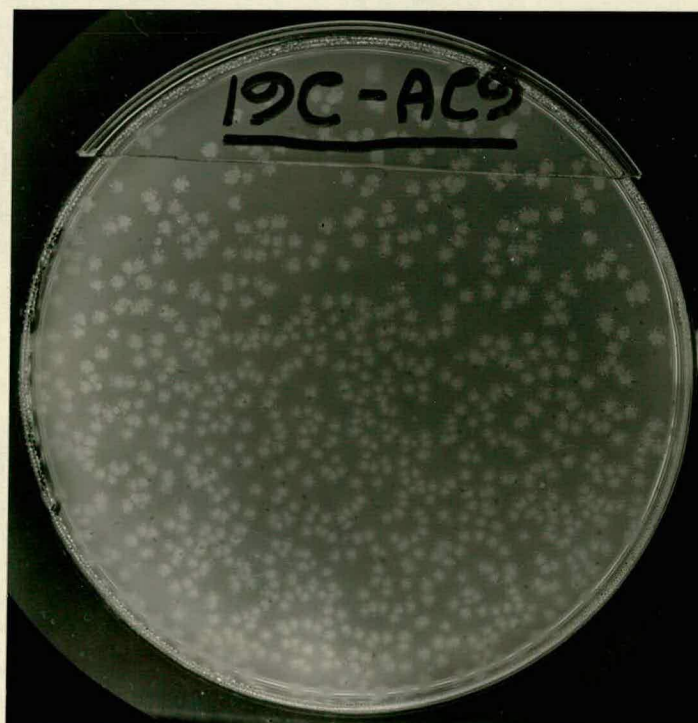
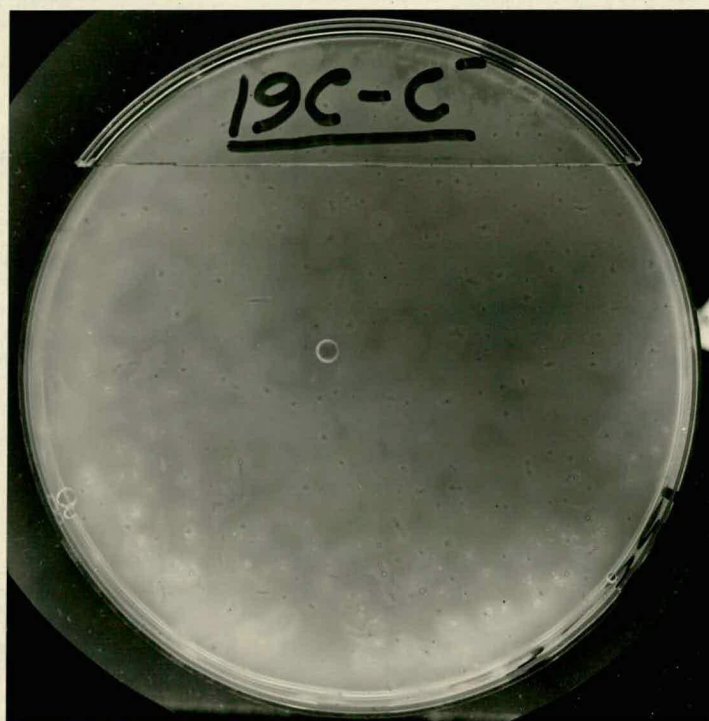
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9



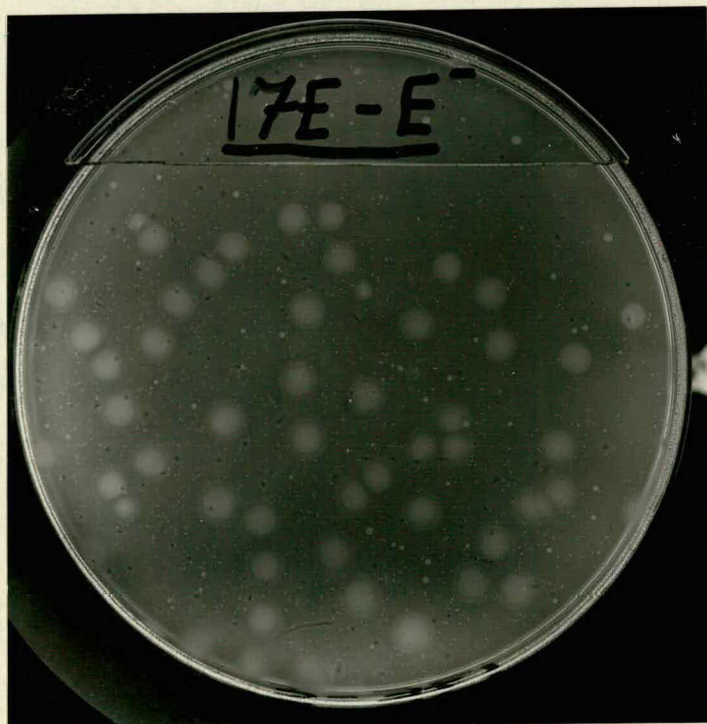
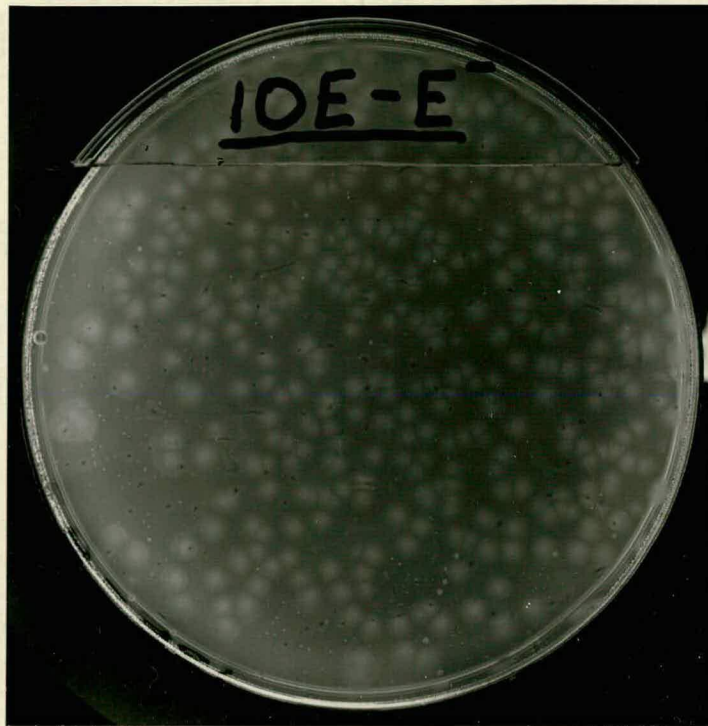
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11



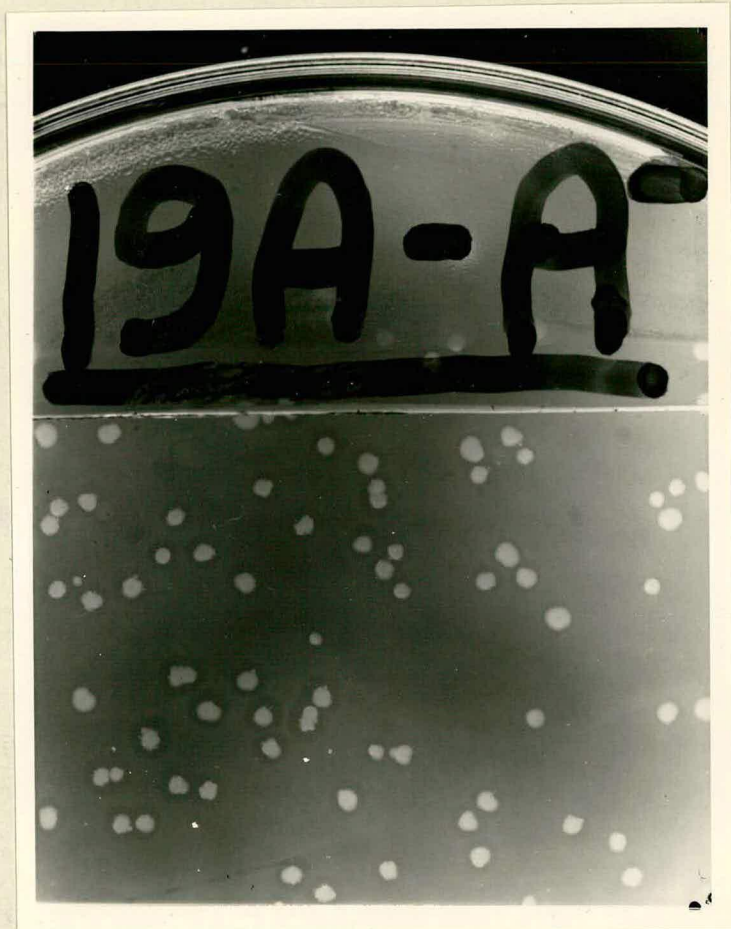
12

13



14

15



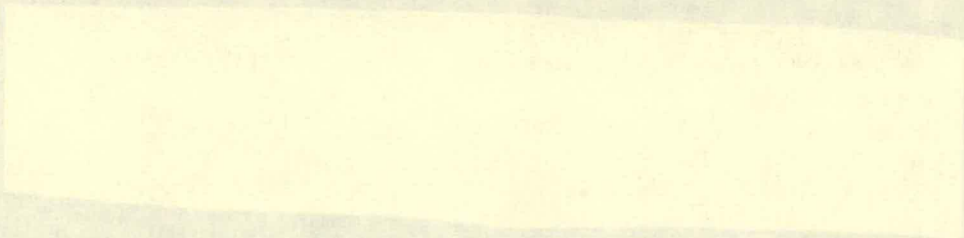


TABLE 1 Trp response of lysates

Lysate No.	<u>Trp</u> phage isolated			Lysate No.	<u>Trp</u> phage isolated		
1	A	C	E	15	A	C	-
2	A	C	-	16	A	-	-
3	-	-	E	17	A	C	E
4	-	-	-	18	A	-	-
5	-	-	E	19	A	C	-
6	A	C	-	20	-	C	-
7	-	-	E	21	A	-	-
8	A	C	E	22	A	C	-
9	A	C	-	23	A	-	E
10	-	C	E	24	A	-	-
11	A	-	-	25	A	C	-
12	A	C	-	26	-	-	-
13	A	-	-	27	-	C	-
14	-	-	-	28	-	-	-

Totals:

18 Trp A phage

14 Trp C phage

8 Trp E phage

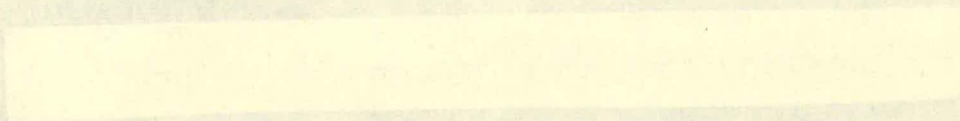
The letters in the columns indicate each type of phage isolated, i.e. lysate 1 yielded a trpA phage, a trpC phage and a trpE phage.

From each of the 28 lysates about 10^6 phage were plated on minimal + ACH plates after adsorption to E. coli strains each with a marker in one of the five trp genes, E, D, C, B and A. The results are presented in table 1. All but four of the lysates yielded at least one type of trp phage. Predominant were phage carrying a single trp gene, trpE, trpC or trpA. From lysate 19, two phage were isolated, one as trpA⁺ and subsequently shown to be trpB⁺ and trpC⁺; one as trpC⁺ and found to carry also trpB and trpA. No phage were isolated complementing cells with lesions in the trpD gene.

The nomenclature of the phage is a number (representing the lysate from which it was isolated), followed by a letter indicating the trp gene it carries.

Genetic characterisation

The structure of the receptor phage with a heterologous insertion of DNA at R.HindIII site 3 is shown in Fig 2. In earlier experiments using the EcoRI enzyme Murray and Murray, (1974), frequently encountered the problem that insertion of DNA fragments, or the deletion of a segment of lambda DNA, (such as that between EcoRI sites 1 and 2), would yield phage which had not arisen by ligation at both ends of the insertion. Frequently phages were found where the EcoRI targets had not been reconstructed. Many of these were integration deficient, some Red⁻, indicating that an illegitimate recombination event had occurred, to circularize the molecule, probably subsequent to transfection. Fig 2 shows how these events may occur. During incubation with the ligase, the probability of the cohesive ends of lambda annealing is much greater than that for the ends created by R.EcoRI, (or R.HindIII),



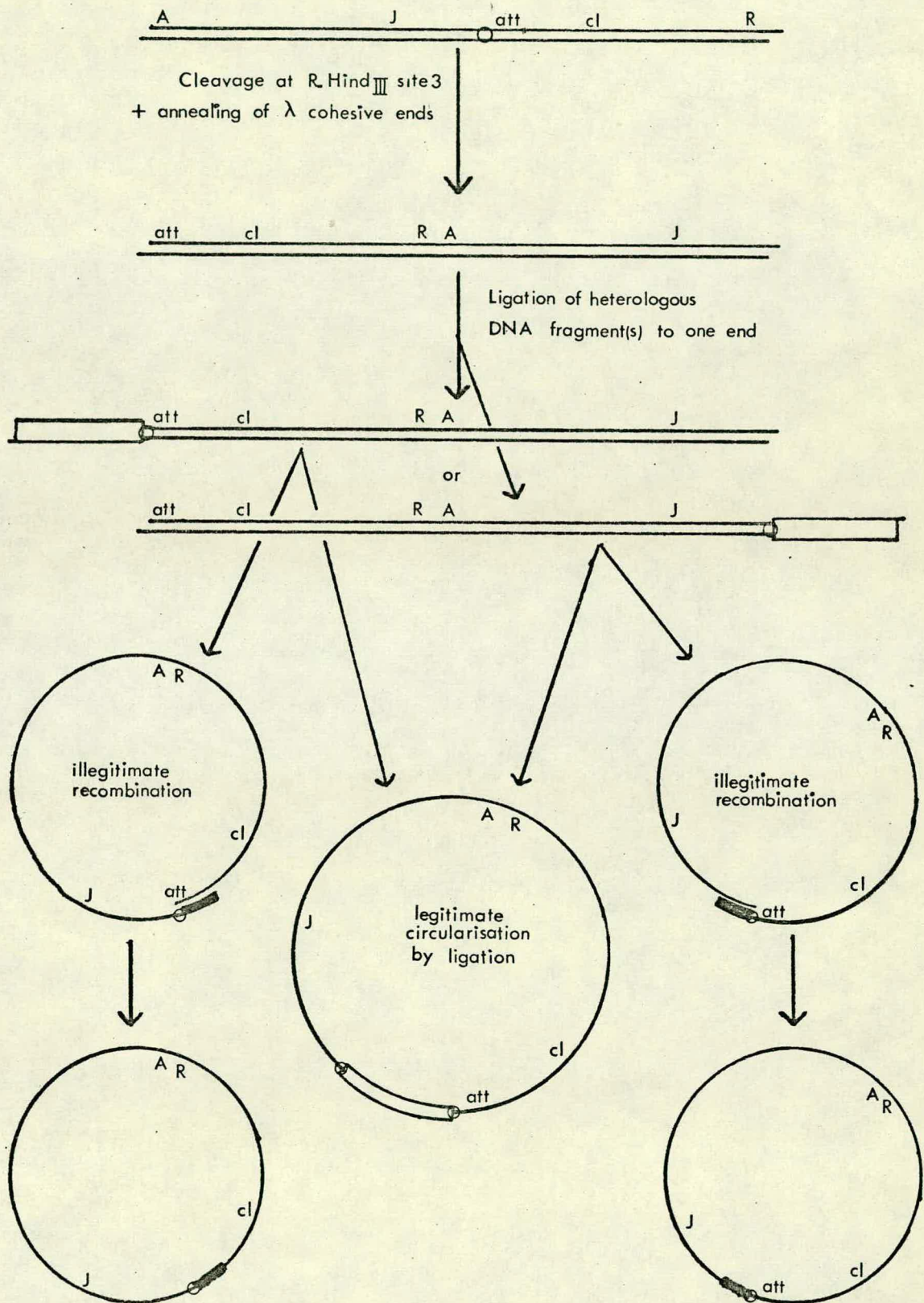
Legend to Fig 2

R.HindIII targets are shown by small open circles. The illegitimate recombinants are deleted for one R.HindIII site, part of the insertion and part of the lambda chromosome.

This is a model for the way these events may occur.

Fig 2

Genesis of 540-trp's



digestion, since there are three times as many complementary bases at the cos site. A molecule with a heterologous DNA fragment attached at one end must circularize upon transfection when it enters the host cell, otherwise it will be degraded by host nucleases.

Test for functional red genes

Phage were tested for their ability to grow on a polA strain, as described in Materials and Methods. Murray, de Ritis and Foster, (1973) demonstrated that phage lacking functional red α or red β proteins have a very low burst size or do not plate at all on strains carrying a polA mutation. All the in vitro trp phage gave a positive response compared to a Red⁻ control phage, so none has lost red function.

Test for integration proficiency

Phage were tested for a functional int gene and attachment site by their ability to form stable, immune lysogens. (The EMB-plate test described in Materials and Methods). They were lysogenised in two different strains, (1) KB30 trp2; a strain carrying a large deletion from the ϕ 80 attachment site, through the tonB gene and all of the trp genes. This means there is no homology for possible Rec or Red mediated integration of the phage into the host trp genes using the homology of the in vitro incorporated fragment. (2) They were also lysogenised in the recA strain QR48, which should prevent recombinational integration of the phage into the trp genes or any other homologous region of the host chromosome.

All the phage were integration proficient except for one: 19C. This phage, one of the two phages isolated from lysate 19, and shown to carry trpC, B and A, was unable to form stable lysogens. The other

trpCBA phage from the same lysate of transfectants, 19A, was integration proficient.

This could mean that these two phage arose quite independently, or that one is a derivative of the other, being unstable due to having inserted too much DNA, and subsequently acquiring a deletion.

Other bacterial markers

The phage were tested to see if they carried the known bacterial markers adjacent to the trp operon. The trpE transducing phage were screened for the cysB gene by plating on strain trpED102 cysB⁻. None of the trpE phage stimulated growth of this strain in the presence of exogenous tryptophan.

The trpA phage were lysogenised in strain KB30 and the lysogens screened for sensitivity to Ø80. This demands the presence of the tonB gene. All the lysogens were resistant to Ø80.

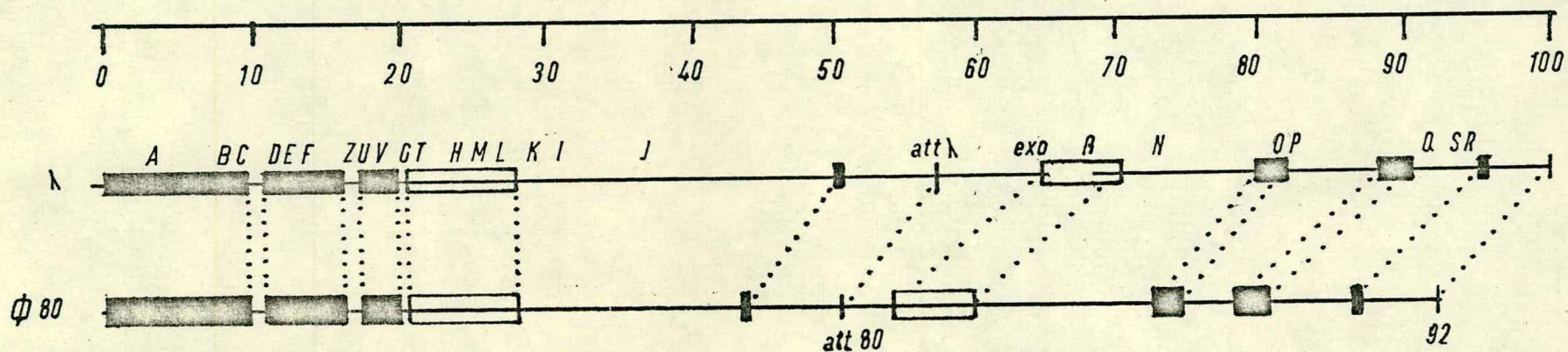
Neither of these results excludes the possibility that the trpE phage could carry part of the cysB gene, or the trpA phage part of tonB.

Determination of the orientation of the transduced genes

Determining the orientation of the trp genes in their novel location was fundamental to the study of their expression. Did the screening procedure for trp⁺ plaques select for phage carrying the trp genes in one orientation only? This could be true for the phage carrying only the A gene since they would not be expected to carry a bacterial promoter

Fig 3

Homology between the DNA's of λ and $\phi 80$



Regions of strong homology represented by closed rectangles,
those of variable homology by open rectangles.

Reproduced from Fiandt, Hradecna, Lozeron and Szybalski
1971

capable of expressing the gene. A terminator in the b₂ region left of the point of insertion of the fragment would not allow transcription rightwards from the late genes to read into the trp gene. Under such circumstances one would not expect to find phages with the gene in that orientation, (see fig 4). For the rest of this thesis, phage with the trp fragment inserted in the same orientation as those of trp transducing phage derived in vivo will be described as carrying the genes in the normal orientation. That is, the sense strand transcribed leftwards, and potentially capable of being expressed from the phage promoter p_L. When the genes are inserted such that the sense strand would be transcribed rightwards in the same direction as the late genes of lambda, they are described as being reversed.

To determine the orientation of the inserted genes a simple and rapid genetic screening procedure was used, fig. 4. It is based on information of the regions of homology between phage lambda and Ø80, provided by the work of Flandt, Hradecna and Szybalski, (1971). Fig 3, from this paper, shows the regions of homology, between these two phages, determined by heteroduplex mapping. As mentioned in the introduction, (p14), phages carrying the attachment site of Ø80, either Ø80 itself or lambda-Ø80 hybrids will infrequently give rise to transducing phage carrying the trp genes substituted into the N-operon of the phage. (Matsushiro, 1963. Deeb, Okamoto and Hall, 1967). (The arrangement of genes on lambda and Ø80 are believed to be very similar although certain gene products of one are not interchangeable or compatible with those of the other (Szpirer, Thomas and Radding, 1969; Szpirer and Brachet, 1970; Deeb, 1970).

The phage Ø80 pt190 carries the whole of the trp operon inserted between the attachment site of the phage and the N gene. This

TABLE 2 Crosses to determine orientation

Test Ø		Recombination Frequency
1A		1.89×10^{-3}
6A		1.7×10^{-3}
8A		2.58×10^{-3}
9A		1.51×10^{-3}
13A	less than	1.3×10^{-6}
17A		3.8×10^{-4}
19A		2.55×10^{-3}
1C	less than	1.13×10^{-6}
2C		6.1×10^{-4}
6C	less than	8.13×10^{-7}
8C	" "	1.4×10^{-6}
9C	" "	8.3×10^{-7}
10C		1.64×10^{-3}
12C	less than	4.8×10^{-7}
15C	" "	5.48×10^{-7}
17C	" "	6.7×10^{-7}
19C		1.08×10^{-3}
20C	less than	5.75×10^{-7}
27C	" "	7.49×10^{-7}
1E	less than	1.04×10^{-7}
3E	" "	7.69×10^{-7}
5E	" "	9.43×10^{-7}
7E	" "	7.49×10^{-7}
8E	" "	6.15×10^{-7}
10E	" "	8.3×10^{-7}
17E		3.9×10^{-4}
540	less than	9.0×10^{-7}
540 x Ø80 wild type		1.78×10^{-5}
11B-1a x Ø80pt190		1.21×10^{-3}

all crosses done
with Ø80pt190
(A⁻, C⁻, or E⁻)
except where stated.

Details of
selection
procedures in
fig 4

substitution therefore removes one of the large regions of homology between Ø80 and lambda. The deletion in the receptor phage 540, between R.EcoRI sites 1 and 2, removes the only other region of homology between the two phages within the region of the genome from gene J (the host range determinant), and the immunity region. In a cross between Ø80pt190 and any of the in vitro constructed trp phage a recombination event between genes J and N should only occur due to the homology provided by the trp genes. If the in vitro trp phage has the trp genes inverted with respect to those on the Ø80ptrp then this homology would not allow a recombination event in trp to produce a viable phage.

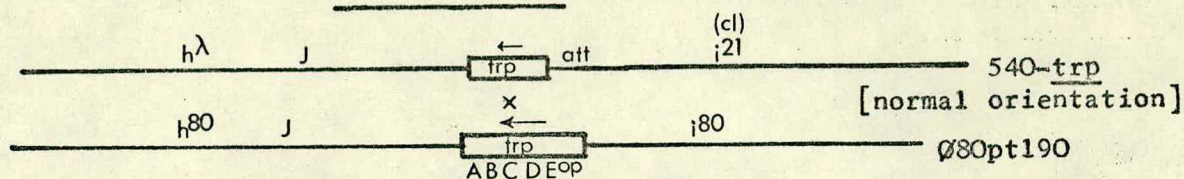
Such crosses were done with each of the in vitro trp phages and table 2 shows the recombination frequency observed in these crosses. There are clearly two classes of phage. Those which gave recombinants at a frequency approaching 10^{-3} , and those for which recombinants could not be detected. Control crosses were done with: a), the receptor phage 540 x Ø80pt190, b) 540 x Ø80 wild type, (where the Red homology is present), and c), 11B-1a x Ø80pt190, another in vitro trp phage constructed by ligation of a trp fragment, cut from an in vivo derived trp transducing phage, joined with ligase to a receptor, (Murray & Murray, 1974), and shown (A.Hopkins, unpublished results) to express the trp genes characteristic of transcription from p_L; (normal orientation).

Recombination between the receptor phage 540 and pt190 was undetectable; between 540 and Ø80 wild type the homology in the vicinity of the red genes allowed a limited amount of recombination; 11B-1a x pt190 gave recombinants at the same frequency as those in vitro

Fig 4

CROSSES TO DETERMINE ORIENTATION

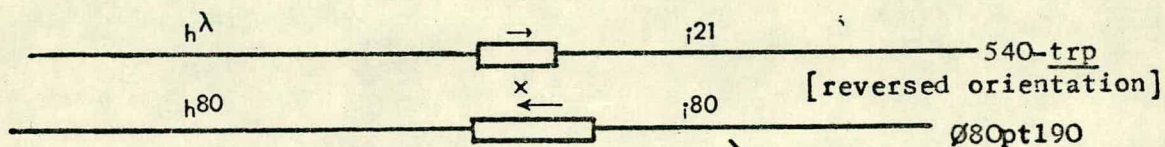
OF TRP GENES



Demanding recombination
in the region J-cl
by selecting $h^{\lambda}i^{80}$
progeny on C600(i^{21})



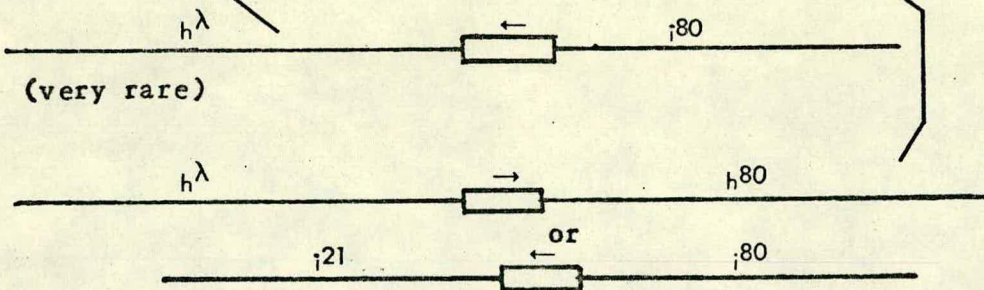
OR



Recombination outside
trp yields:

h^{λ}
(very rare)

Recombination in
trp yields:



(inviabile phage molecules)

constructed trp phage of normal orientation (fig 4 shows the crosses and gives details of the selection procedures imposed).

The results of this experiment allowed the selection of phages for more detailed study. The phages were divided, on the basis of the trp genes they carried and their orientation into seven groups. One representative from each group was selected for more detailed investigation. These representatives were:

9A	carrying	<u>trpA</u>	in the normal orientation
13A	"	"	" " reversed orientation
10C	"	<u>trpC</u>	" " normal orientation
8C	"	"	" " reversed orientation
17E	"	<u>trpE</u>	" " normal orientation
10E	"	"	" " reversed orientation
19A	"	<u>trpA</u> , <u>B</u> and <u>C</u>	in the normal orientation

The phages 13A and 17E are the sole representatives of their respective classes. The trpA and trpE phage show a strong, non-random bias towards a particular orientation. Six out of seven of the trpA phage are orientated so they could be transcribed from the phage promoter. p_L . This may be because this fragment does not carry any known bacterial promoter and so must rely on a phage promoter for its expression. The fact that a phage carrying this fragment, reversed, was found, suggests strongly that there must be rightward transcription from a phage promoter reading the gene; either from p_R' or from a promoter in the b_2 region. As can be seen from plate 2, the trp⁺ plaques produced by phage 13A are different and much weaker than those of any of the other phages. This may reflect a very low level of expression of the trpA gene.

Six out of seven of the trpE carrying phage have the fragment reversed. Since this fragment carries the trp promoter (as will be shown conclusively later), and is thus capable of being expressed independently of any phage promoter, it is not obvious why there should be any selection for a particular orientation.

Confirmation of Orientation by Heteroduplex Mapping

If the main conclusion of the previous experiment was correct then DNA:DNA heteroduplexes between phages carrying the same fragment, but in opposite orientations, should show a region of non-homology corresponding to the inversion. If the phage carry the same fragment, cut from the trp operon by the R.HindIII enzyme, then the regions of single stranded non-homology should be of equal length.

Plates 16-18 are of heteroduplexes between phages 9A and 13A; 10C and 8C; 17E and 10E. They show that the above prediction is confirmed and that the regions of non-homology are of equal size, for each pair of phages.

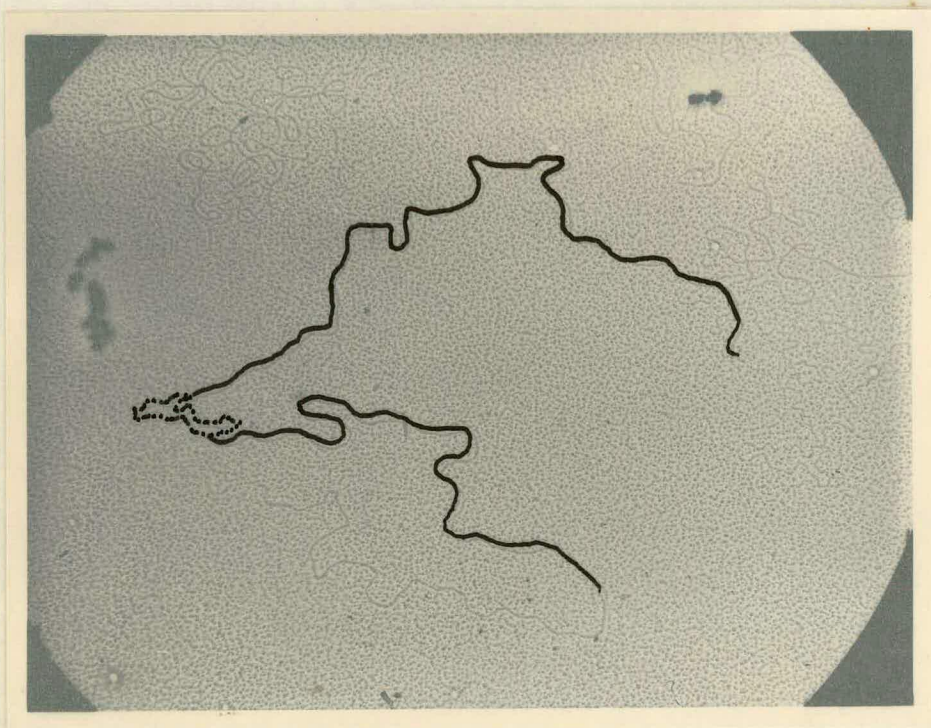
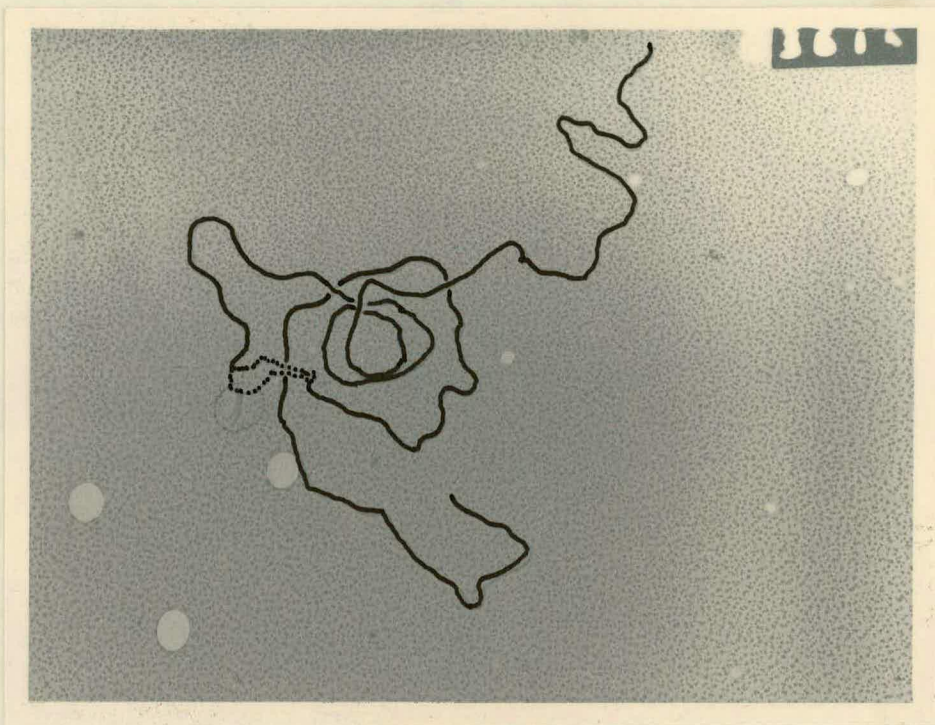
The extent of the trp operon carried by the phages

The end points of the transduced fragments were of interest for two reasons. In the first place: were the three types of insertions a sequence of fragments from the trp operon? That is; did the trpA fragment end somewhere in the trpB gene at a point where the trpC fragment began, (and similarly for the trpC and trpE phages with end points in trpD), or were there two targets for the R.HindIII enzyme in trpB and/or trpD? Secondly, the endpoints of the fragments should, if they were inserted by ligation of the cohesive ends created by R.HindIII, define at least two targets for this enzyme in the trp operon.

Plate	Phages Heteroduplexed
16	<u>9A</u> against <u>13A</u>
17	<u>8C</u> against <u>10C</u>
18a & b	<u>10E</u> against <u>17E</u>

In the tracings, double stranded regions of homology
are represented by continuous lines: single stranded
regions of non-homology as dotted lines.

16



17

18a

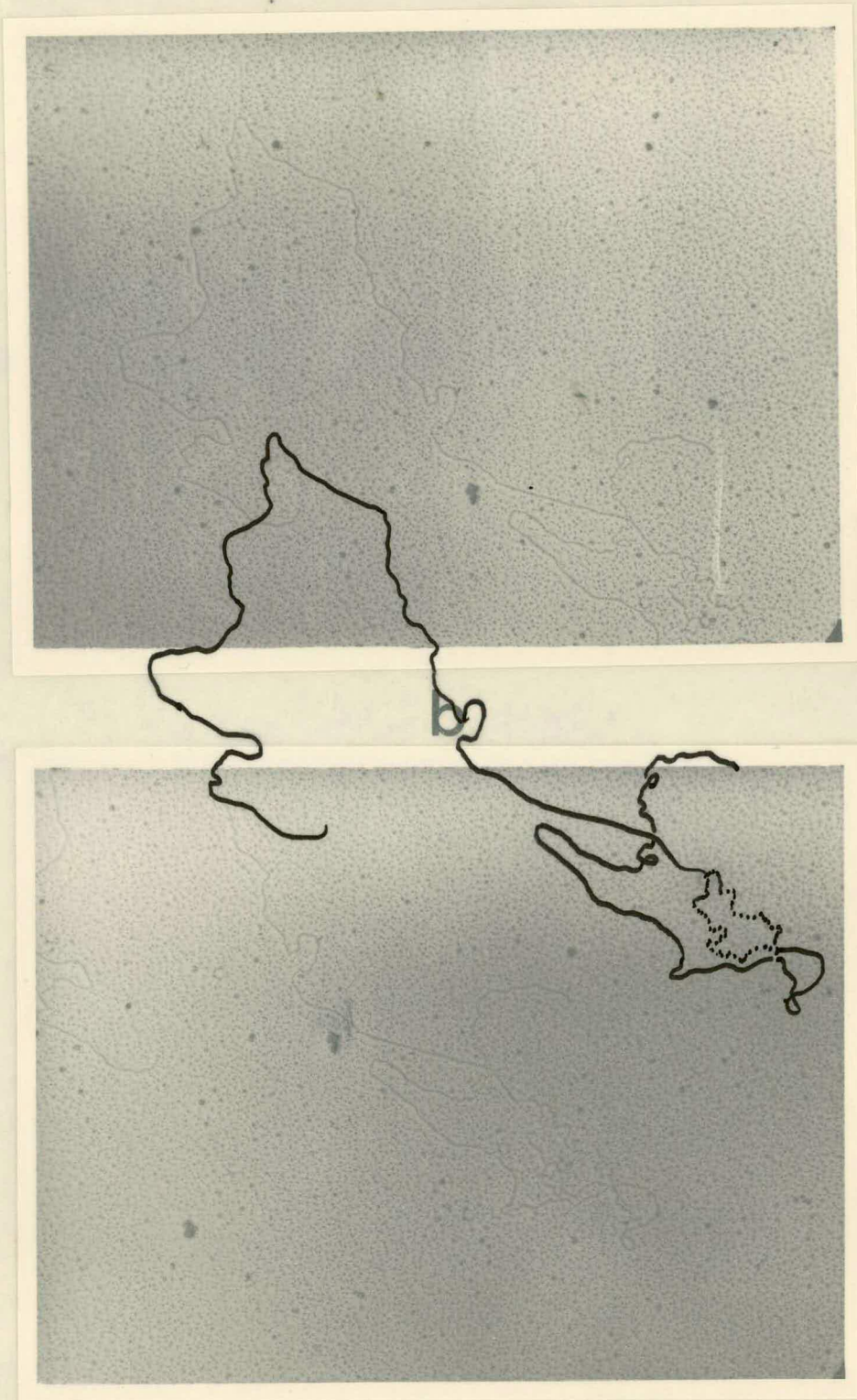
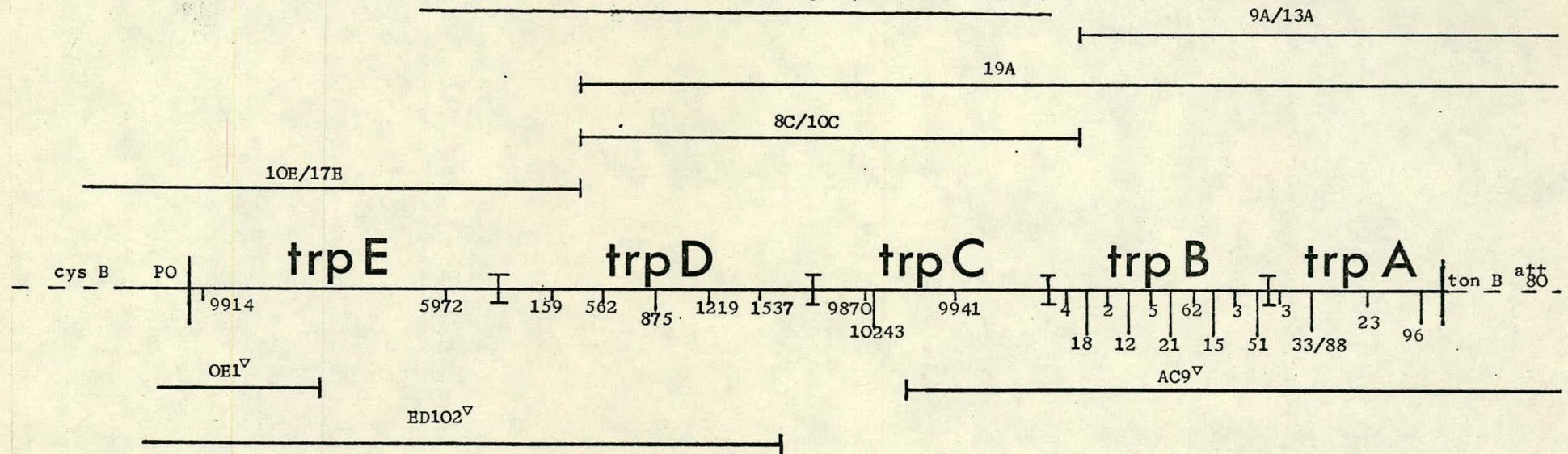


Fig 5

The Extent of the TRP Operon Carried by the Phages,
and the Mutations used for mapping by Marker Rescue.



A series of points mutants in the trpB and trpD genes (fig 5) were used to ask whether the wild type allele could be rescued from the trp fragment carried by a particular phage.

The phages 9A and 13A both transduced all the markers up to and including B18 at the promoter proximal end of the trpB gene, but not the more proximal mutation B4. The phages 10C and 8C both transduced B4 but not B18. In the trpD gene, phages 10C and 8C transduced all markers up to and including D562 at the promoter proximal end, but not D159. Phages 17E and 10E transduced D159 but not D562. The phage 19A which complements the deletion AC9 ending in trpC transduced the same markers in trpD as phages 10C and 8C.

From this it is likely that there are only two targets in the trp operon for the R.HindIII enzyme. The transducing phage could have cloned single adjacent fragments from trp, and phage 19A probably carries two adjacent fragments, the product of incomplete digestion with the restriction endonuclease.

Ability of the phages to express their trp genes

in an immune cell

a) Bacterial strains carrying a lesion in one of the five trp genes were lysogenised with an in vitro phage which transduced that gene, (eg. W3110 trpE₉₅₇₂ with 17E or 10E, etc). Stable lysogens all became prototrophic and so must have some means of expressing the trp gene on the phage. It is possible that the phage could have lysogenised by recombination into the host trp operon and the gene be expressed from the trp promoter. (This is unlikely in the presence of functional attachment sites.)

TABLE 3

EXPRESSION OF THE PHAGES AS LYSOGENS

Lysogen	Phage Ø80pt190				
	A ⁻ (BCDE) ⁺	B ⁻ (ACDE) ⁺	C ⁻ (ABDE) ⁺	D ⁻ (ABCE) ⁺	E ⁻ (ABCD) ⁺
KB30 (9A)	+	-	-	-	-
KB30 (13A)	+	-	-	-	-
KB30 (19A)	+	+	+	-	-
KB30 (8C)	-	-	+	-	-
KB30 (10C)	-	-	+	-	-
KB30 (17E)	-	-	-	-	+
KB30 (10E)	-	-	-	-	+

+ means the combination results in the
formation of trp⁺ plaques

- a negative response

b) Lysogens were constructed of each phage in strain KB30 trp2, which is deleted for the region trp through tonB and att80. These lysogens were superinfected with Ø80pt190 phages of extended host range (thus able to adsorb to a tonB cell). Ø80pt190 carries the whole trp operon and such phages, with mutations in each of the five trp genes, were plated on the lysogens. Trp⁺ plaques appeared only when the super-infecting pt190 phage was mutant in the gene carried by the in vitro trp phage in the lysogen. (Table 3).

This confirms that all the in vitro trp phages can express their incorporated trp gene irrespective of orientation and of the presence of phage repressor. Phages 17E and 10E probably carry the trp promoter allowing them to express trpE subject to the influence of the trp repressor. Phages 10C and 8C should carry the constitutive promoter, late in the trpD gene and so they too would be able to express their gene in a lysogen. Phages 9A and 13A do not have any known promoter. If 9A were a double lysogen could be expressed from the leftward reading constitutive promoter in the phage xis gene (Shimada, Gottesman & Weisberg, 1973). Phage 13A could not express by repressor independent read-through rightwards from p_R' (nor by transactivation of the 13A p_R' by the Ø80 Q protein, (Q product from Ø80 cannot transactivate to initiate transcription from p_R' of lambda). The only explanation is a constitutive promoter in the b₂ or late gene operon region, left of R.EcoRI site which reads into the trp gene of this phage.

U.V. inducibility of lysogens

Lysogens of phages 9A, 13A, 8C, 10C, 10E and 17E in strain KB30trp2 were subjected to U.V. irradiation (450 ergs/mm^2) and the induced phage assayed as described in Methods under "Comparative U.V. inducibility of lysogens." Controls included were non-lysogenic KB30trp2 and KB30trp2 lysogenised with the parental receptor phage 540.

From an initial cell concentration of 3×10^8 /ml all the lysogens produced phage at a concentration varying from 8×10^9 /ml to 4×10^{10} /ml.

From this it can be concluded that none of the phages possess any deficiency in the excision process.

Physical Characterisation

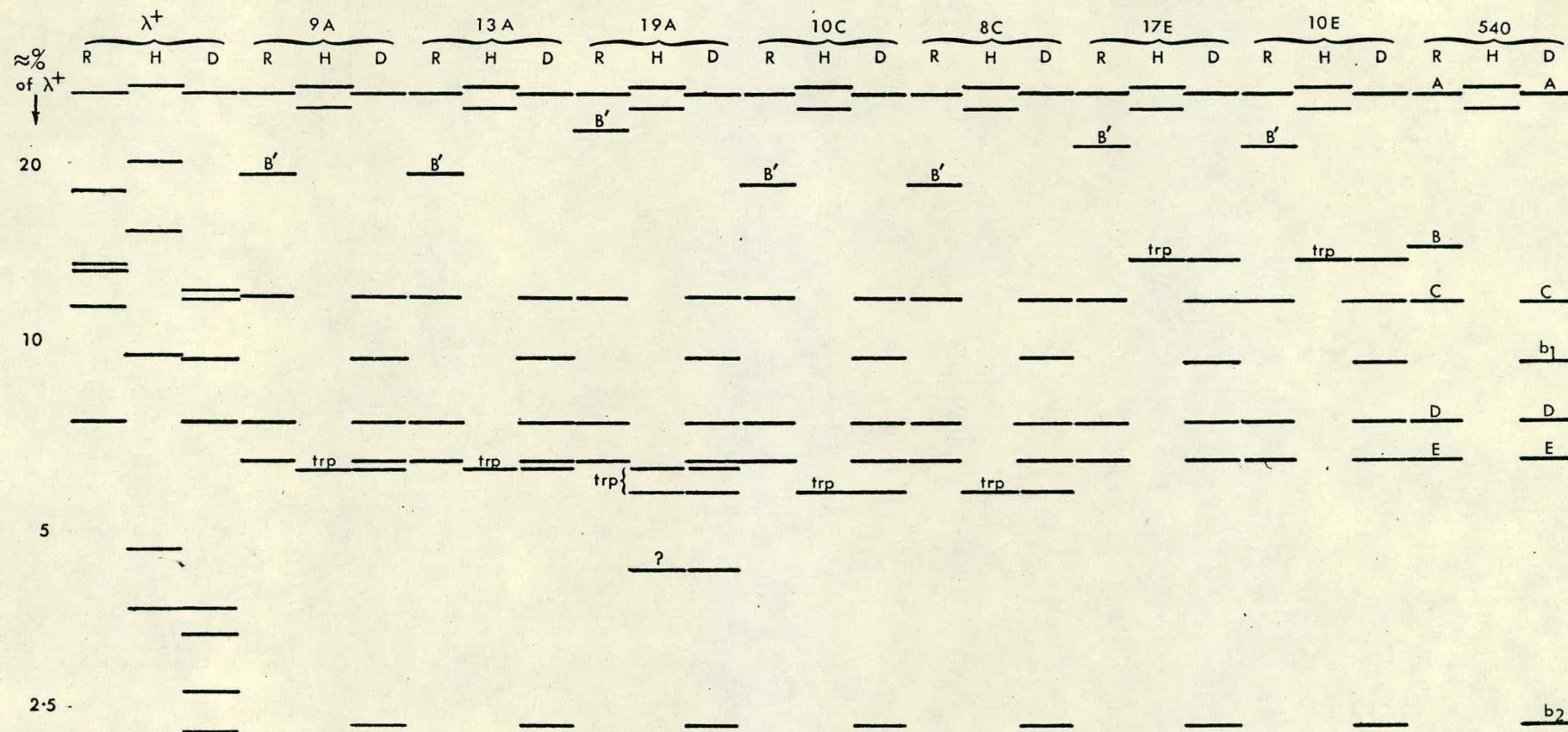
The results of the previous experiments suggested that the three basic types of transducing phages had incorporated single fragments cut from four HindIII targets in the E. coli chromosome; two in the trp operon itself and two outside, one in or near the tonB gene and one located in the vicinity of the cysB gene. It was not possible to map genetically the positions of these other two targets due to the paucity of mutations in these genes. However, if the fragments had been inserted into the receptor phage by hydrogen bonding and subsequent ligation of the cohesive ends created by the restriction enzyme, then two new R.HindIII targets should have been created in place of the single original one in the receptor phage. Restriction of the phage DNA with R.HindIII enzyme followed by separation of the fragments on agarose gels should resolve at least three fragments. The left and right arms of the receptor phage should be visible, (46.6% and 33.1% of the size of wild type lambda, respectively), plus at least one other fragment, corresponding to the insertion. The size of the extra fragment can be calculated, using the fragments of wild type lambda digested with R.EcoRI as standard size markers.

Plates 19 to 23 show digests of the seven phage DNA's, run on agarose gels, together with phage 540, and lambda wild type DNA. Fig 6 is a collation of all the photographs with the positions of the fragments of each phage drawn so as to allow comparison between them. The sizes of the insertions are, (in terms of percentage size of wild type lambda), 11.5% for the trpE fragment, 6.1% for trpC and 6.6% for trpA. Phage 19A carries two fragments corresponding to trpC and trpA but in addition has a smaller fragment of 4.0%.

Legend to fig 6

Fig 6 was constructed using the data from plates 19-23. The molecular sizes of the R.EcoRI digest of lambda wild type (track 1) are, from top to bottom, i: 44.5%; ii: 15.4%; iii: 12%; iv: 11.3%; v: 9.8%; vi: 7%, of wild type lambda.

The band with a question mark in the 19A digests is the extra fragment to which no function has yet been ascribed.



Collation of data from agarose gels

[Digested with R - R.EcoRI
 H - R.HindIII
 D - digested with both enzymes]

Fig 6

Plate 19

Key

Left to right the tracks are:

- (1) Undigested lambda wild type DNA
- (2) R.EcoRI digest of lambda wild type
- (3) R.HindIII digest of lambda wild type
- (4) R.EcoRI + R.HindIII double digest of above
- (5) R.EcoRI digest of receptor phage 540
- (6) R. HindIII digest of receptor phage 540
- (7) R.EcoRI + R.HindIII double digest of 540
- (8) R.EcoRI digest of phage 10E
- (9) R.HindIII digest of phage 10E
- (10) R.EcoRI + R.HindIII double digest of 10E

19



PLATE 20: Key

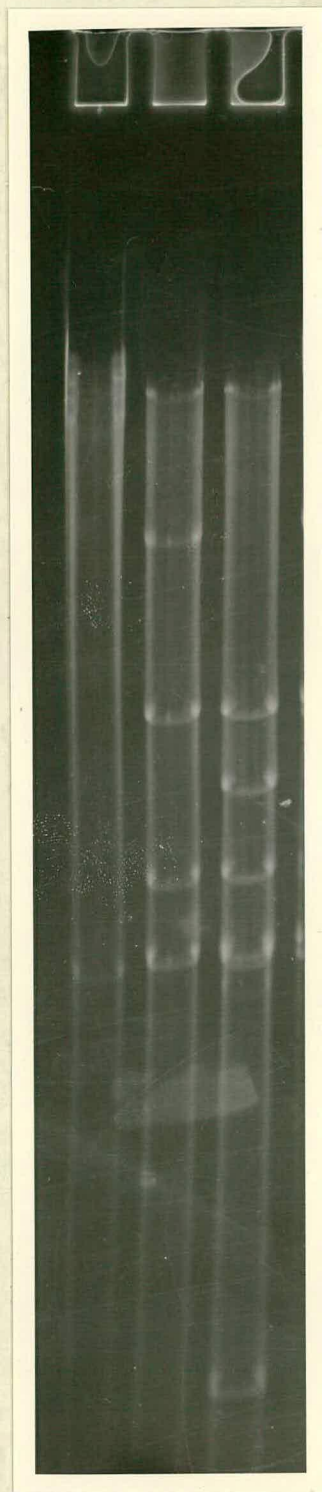
Track

1. R.HindIII digest of phage 9A
2. R.EcoRI " " " "
3. R.EcoRI + R.HindIII double digest of 9A

PLATE 21: Key

1. R.EcoRI digest of phage 19A
2. R.HindIII " " " "
3. R.EcoRI + R.HindIII double digest of 19A

20



21

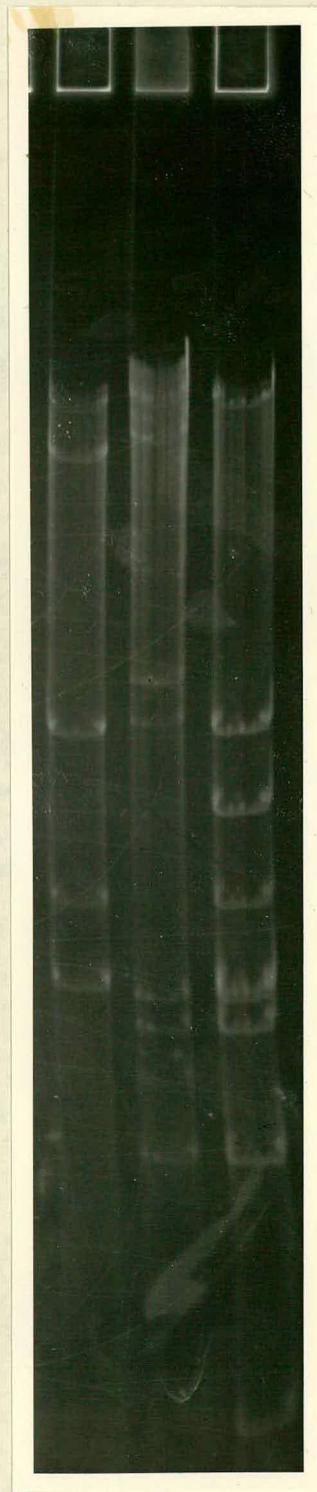


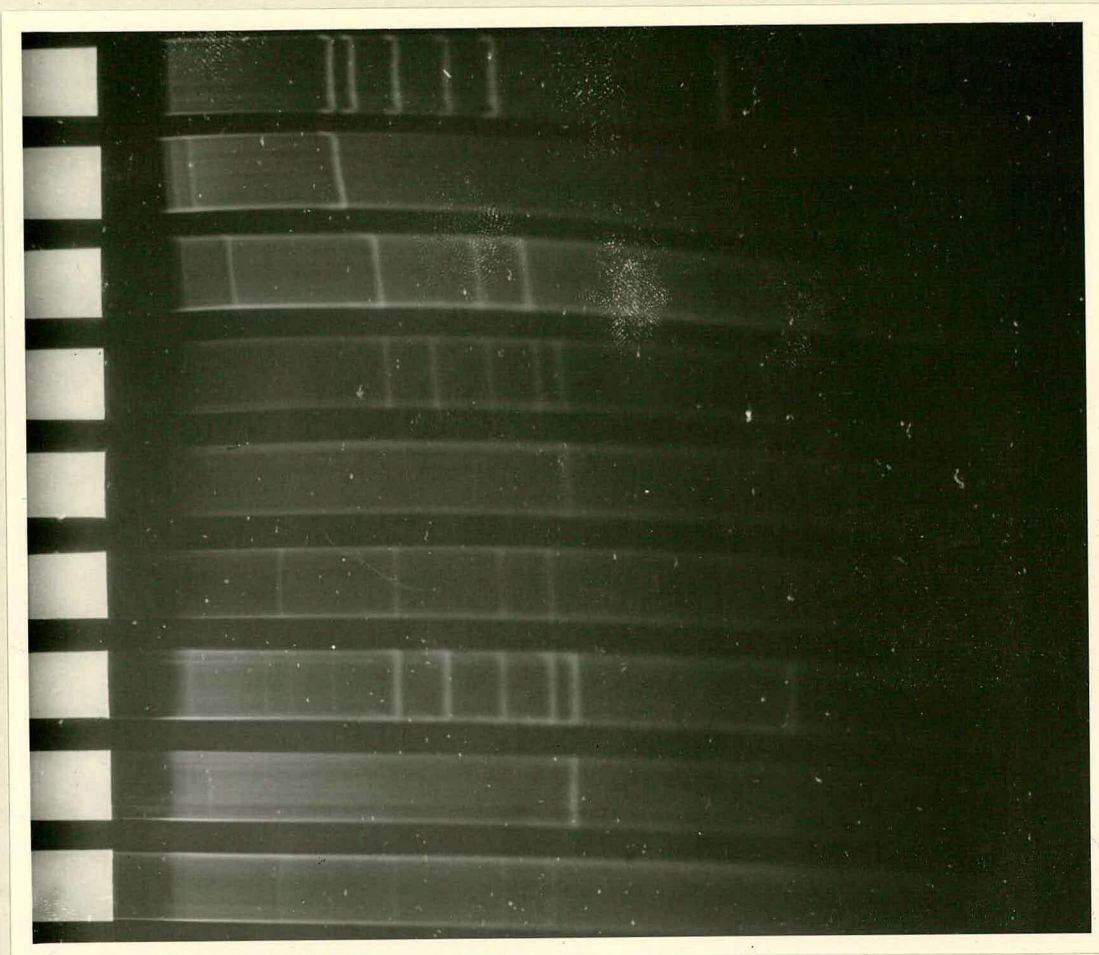
PLATE 22: Key

Track

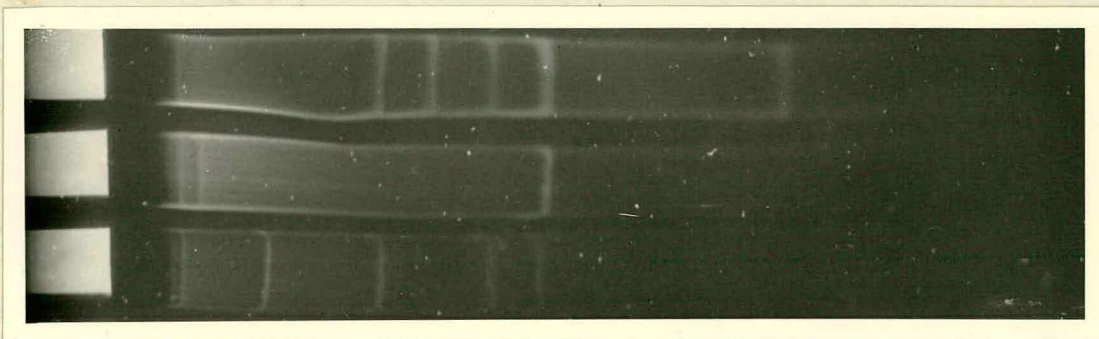
1. R.EcoRI digest of phage 13A
2. R.HindIII " " " "
3. R.EcoRI + R.HindIII double digest of 13A

PLATE 23: Key

1. R.EcoRI digest of phage 10C
2. R.HindIII " " " "
3. R.EcoRI + R.HindIII double digest of 10C
4. R.EcoRI digest of phage 8C
5. R.HindIII " " " "
6. R.EcoRI + R.HindIII double digest of 8C
7. R.EcoRI digest of phage 17E
8. R.HindIII " " " "
9. R.EcoRI + R.HindIII double digest of 17E



23



22

All the phage were digested with R.EcoRI, R.HindIII and with both enzymes together. R.HindIII allows the size of the insertion to be calculated directly; R.EcoRI indirectly, if there is no R.EcoRI target in the incorporated fragment, from the change in mobility of the fragment generated by cleavage at the RI 1/2 (hybrid) target and RI site 3 in the red β gene. This, the fragment containing the R.HindIII target where the E. coli DNA fragment is inserted, experiences an increase in size which should correspond to the size of the insertion. If it is larger than would be predicted then it is likely that the insertion was of two R.HindIII fragments, one of which was cleaved by an illegitimate recombination event. With all of the phage, the R.EcoRI fragment B (Fig 7) corresponds to the fragment B from phage 540 plus a fragment inserted at R.HindIII site 3 equal to the size determined from the R.HindIII digest.

The double digest with R.EcoRI and R.HindIII substantiates this at a more sensitive level. The double digest cleaves the R.EcoRI fragment B into 3 smaller pieces. The insertion at the R.HindIII site plus the two fragments from either side. The fragment b_1 (Fig 7) is 2.9% the size of lambda; b_2 9.0%, (Murray & Murray, 1975). Again any insertion by an illegitimate event would be noticed as a change in the mobility of one of these fragments. At the level of the size of b_1 , differences in size of 0.1% (46 base pairs) can be discerned easily. No such differences are apparent.

These results therefore suggest strongly that all of these phages have acquired R.HindIII fragments from E. coli by hydrogen bonding and subsequent ligation of the R.HindIII generated cohesive ends.

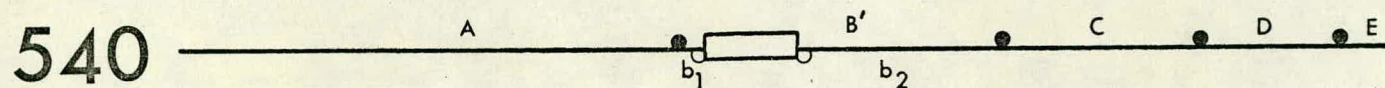
Legend to fig 7

Fragment A corresponds to fragment i of the R.EcoRI digest of lambda wild type; fragment B to iv; fragment E to vi. Fragment C is ii less the difference between imm^{21} and imm^λ , $15.4 - 4.9 = 10.5\%$. D is iii less the ninR_5 deletion, $12 - 5.4 = 6.6\%$.

Fragment b_1 from the double digest 2.9%; b_2 9%.

$B = b_1 + B + b_2$ only if an insertion has occurred legitimately at R.Hind_{III} site 3. Otherwise b_1 or b_2 from the in vitro constructed transducing phage would be larger than b_1 or b_2 from the parent 540.

Fig 7
Fragment nomenclature



+ insertion of heterologous DNA at Hind_{III} site 3

• R.EcoR_I sites
○ R.Hind_{III} sites

These digests also show that there are no R.EcoRI targets in any of the inserted DNA fragments. No extra bands appear in the R.EcoRI digests of the phage. This is in agreement with the results of Brammar, Murray & Winton (1974).

Confirmation of the size of the insertion by E.M.

heteroduplex mapping

Heteroduplexes between the receptor phage 540 and any of the in vitro constructed trp phages should give rise to a double stranded DNA molecule with a single stranded deletion loop originating at the position of R.HindIII site 3 on phage 540.

An ancestor of phage 540, phage 426, carries the immunity of phage lambda, includes the R.EcoRI sites 1 to 2 deletion, but does not carry the ninR₅ deletion. Thus a heteroduplex of 426 with one of the in vitro trp phages (immunity phage 21 and ninR₅ deleted), should show, in addition to a deletion loop corresponding to the trp insertion, a region of single stranded non-homology between the imm²¹ and imm^λ immunities and a deletion loop at the position of ninR₅, near the right hand end of the heteroduplex DNA molecule. The molecule can immediately be orientated from the position of ninR₅. The single strands of known length, imm²¹, imm^λ and nin can be used to calculate the length of the trp insertion. (The non-homology of imm²¹ with imm^λ extends from 71.0 to 79.8% on the lambda vegetative map. Thus the single strand length of imm^λ is 8.8%, (Davidson and Szybalski, 1971). imm²¹ is 4.9% shorter than imm^λ, (Westmoreland, Szybalski & Ris, 1969).

10-438

TABLE 4. SIZE OF TRP INSERTIONS

Heteroduplex	Size (% of λ)
9A:426	7.2
	5.7
	5.37
	5.9
	5.56
13A:426	5.25
	5.9
	5.06
19A:426	16.4
	15.9
	16.2
	16.78
8C:426	5.66
	6.0
	10.83
10C:426	5.4
	9.76
	5.75
	6.0
	9.3
	6.1
	6.35
10E:426	11.7
	11.4
	11.7
17E:426	11.18
	12.0
	11.26

The length of the trp insertion
[x] , is given by:

$$[x] = \frac{X \cdot \gamma}{A+B+C}$$

where:

X = measured length of trp

A = " " " $\frac{1}{21}$

B = " " " $\frac{1}{\lambda}$

C = " " " $\frac{ninR_5}{5}$

γ = actual length of

$$(\frac{1}{21} + \frac{1}{\lambda} + \frac{ninR_5}{5}),$$

as a % of λ wild type,

18.3%

Plates 24-30 show the best examples of a heteroduplex of each of the seven phages. Table 4 gives the sizes of the insertions calculated from the internal single strand standards. The sizes are in fairly close agreement with those determined by agarose gel electrophoresis. There is significant variation for some of the measurements which may be due to hyperextension of one of the single strands used as standards. In addition one or two heteroduplexes of the phages 8C and 10C with 426, (and possibly 9A), have lengths which are far in excess of that expected by variation due to spreading conditions. This could be explained by the trp phages having acquired duplications or insertions to try and restore their DNA content (86%) to normal. On the other hand, some of the 426 phages could contain a deletion covering R.HindIII site 3.

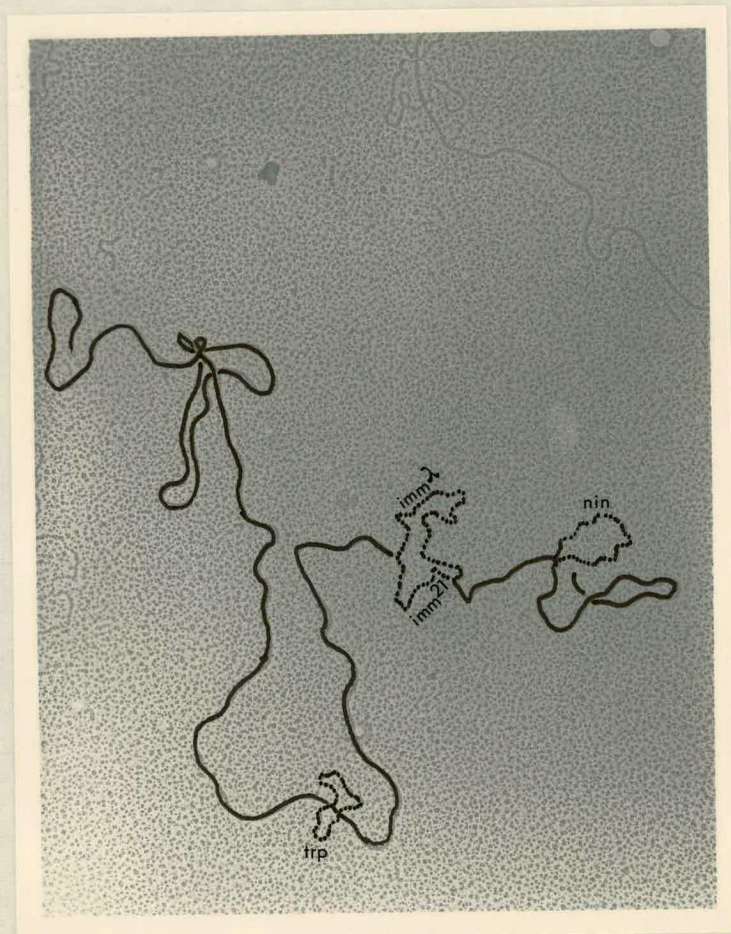
Kellenberger-Guyer, (1971) mentions the observation that lambda b₂ deleted phage and other mutants with large deletions frequently evolve more dense phage during growth. Duplications in lambda are highly unstable in the presence of a recombination system, (Bellett, Busse & Baldwin, 1971; Emmons, 1974; Feiss & Campbell, 1974), but phage with near to wild type size may have a selective advantage, over deletion mutants, under normal growth conditions. Phages 9A, 8C and 10C may well have acquired duplications of their trp DNA using the homology provided by the two R.HindIII targets in the phage.

In vitro construction of 540-trpEDC phage

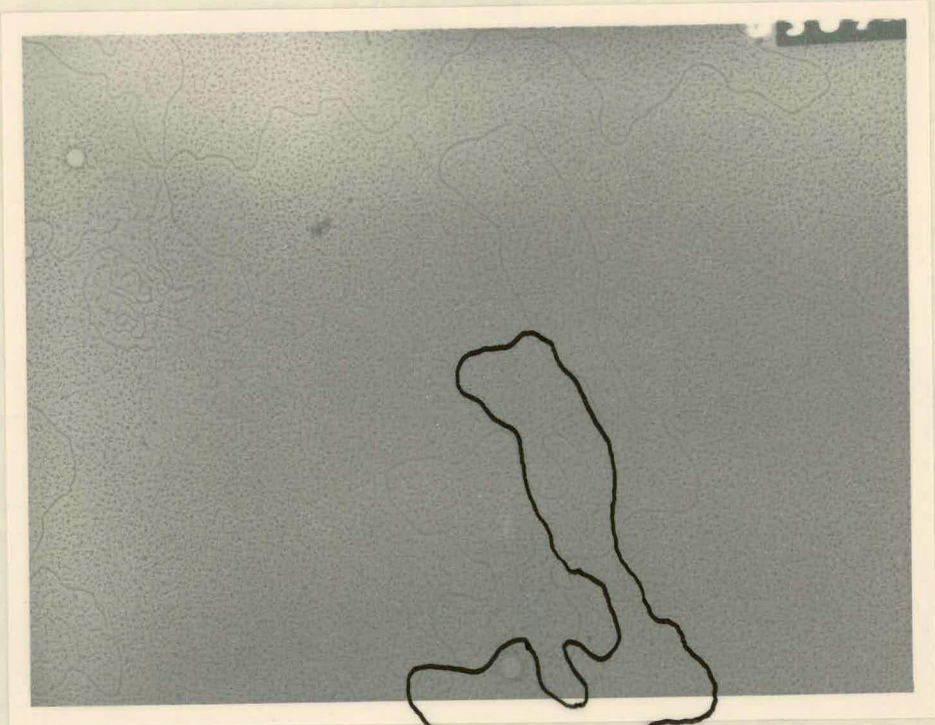
DNA of phages 17E and 10C were restricted with R.HindIII, ligated and transfected. The transfectants were screened for phage which could complement the host trpED102. This strain has a deletion of trpE and

<u>PLATE</u>	<u>PHAGES</u> <u>HETERODUPLEXED</u>
24	<u>9A</u> vs 426
25a &b	<u>13A</u> vs 426
26a &b	<u>19A</u> vs 426
27	<u>8C</u> vs 426
28	<u>10C</u> vs 426
29	<u>10E</u> vs 426
30	<u>17E</u> vs 426

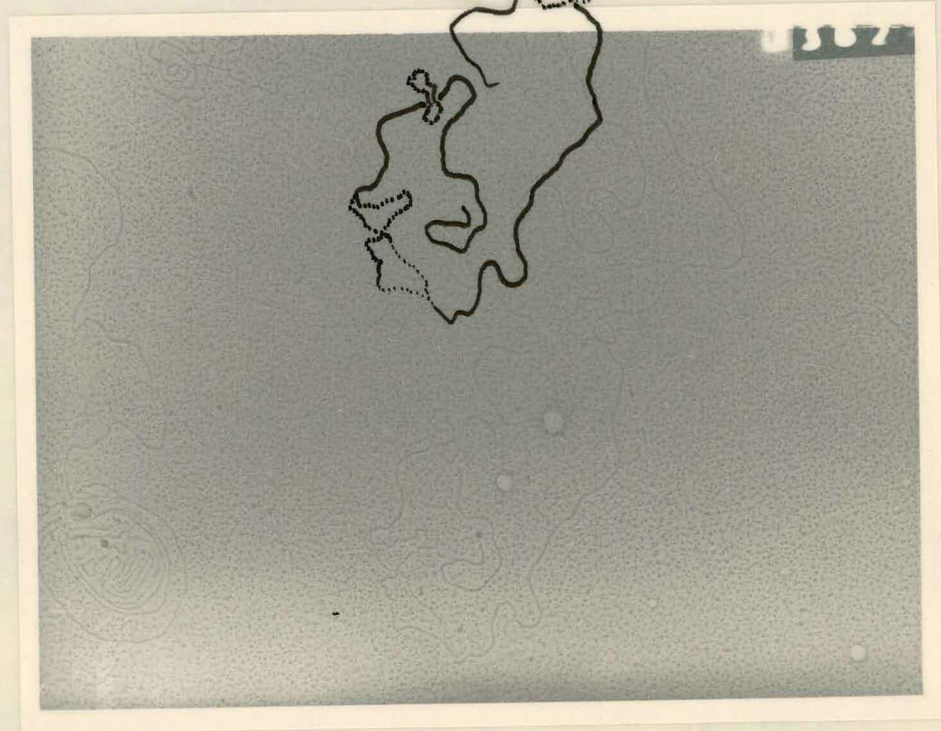
Double stranded regions of homoduplex are represented in the tracings as continuous lines; single stranded regions of non-homology are shown by dotted lines.



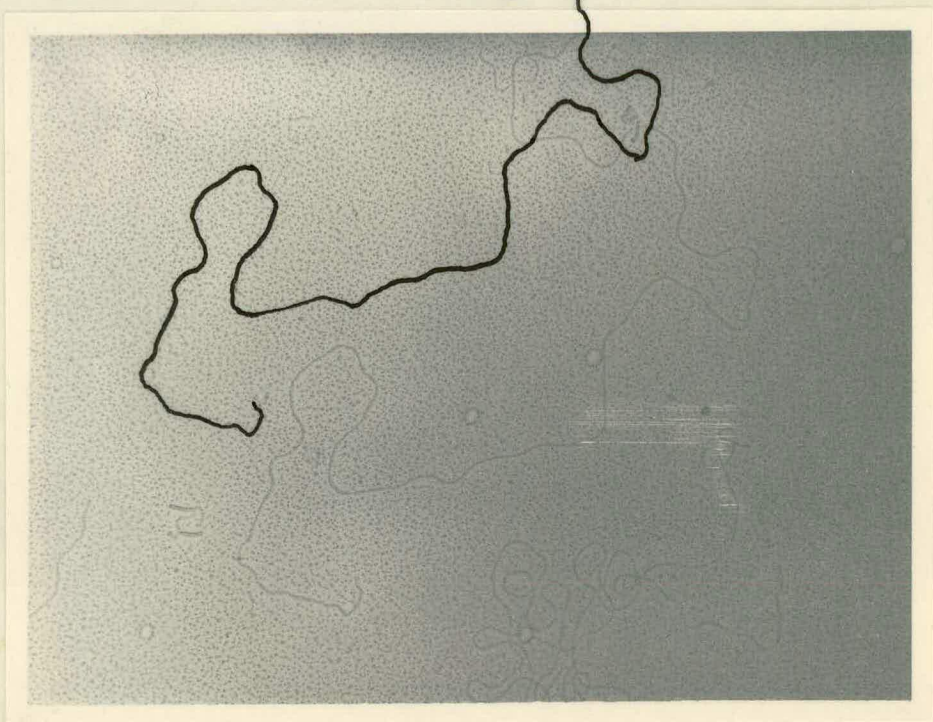
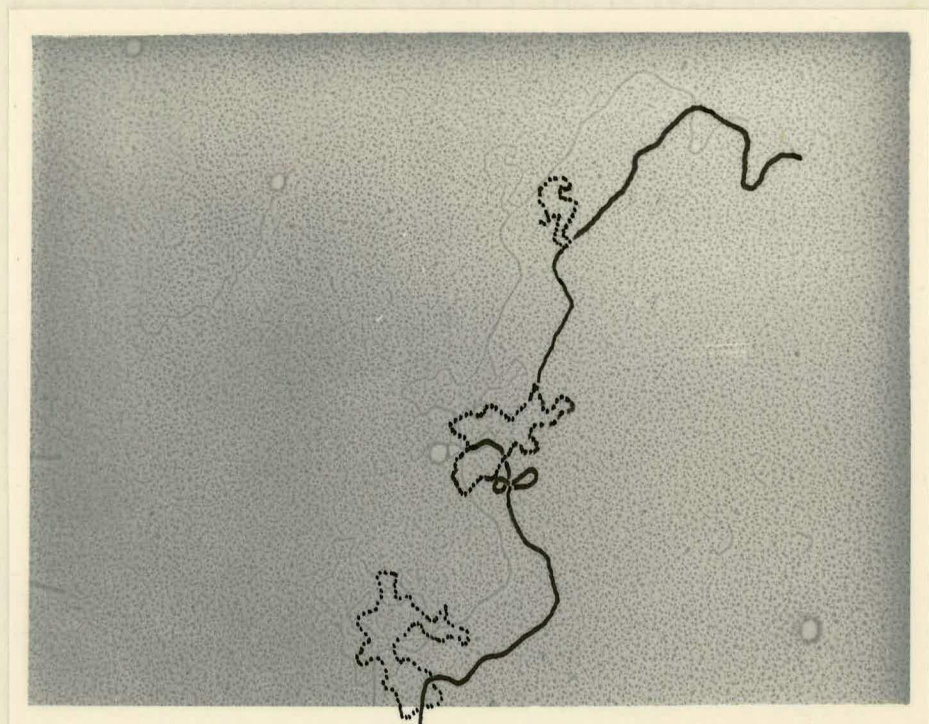
25a



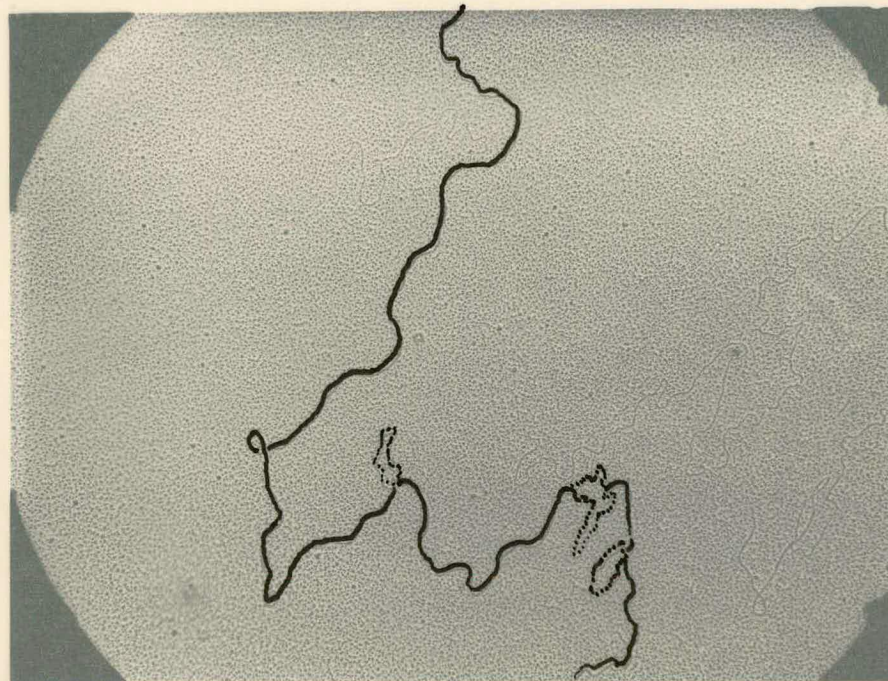
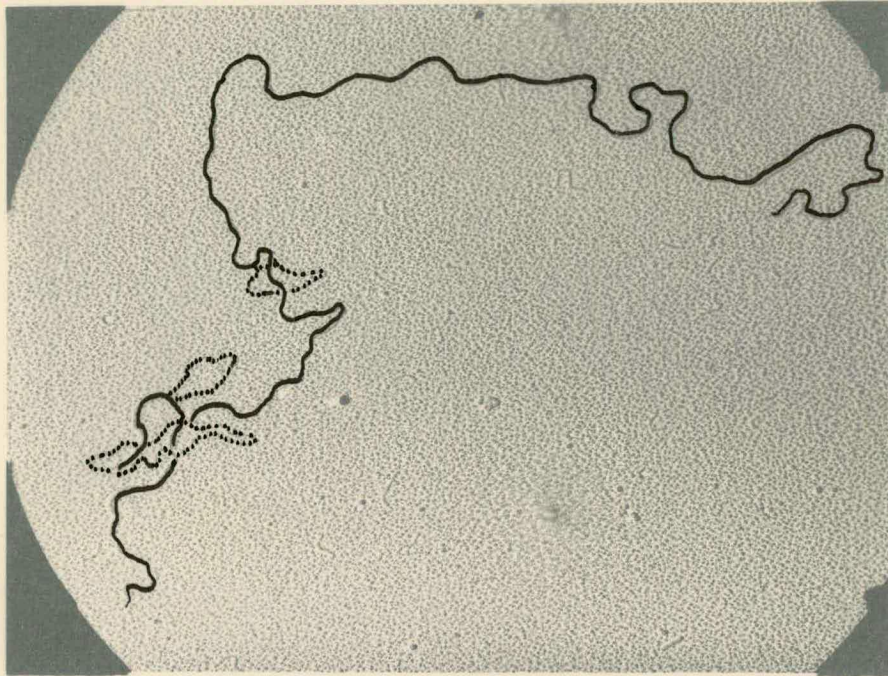
b



26a

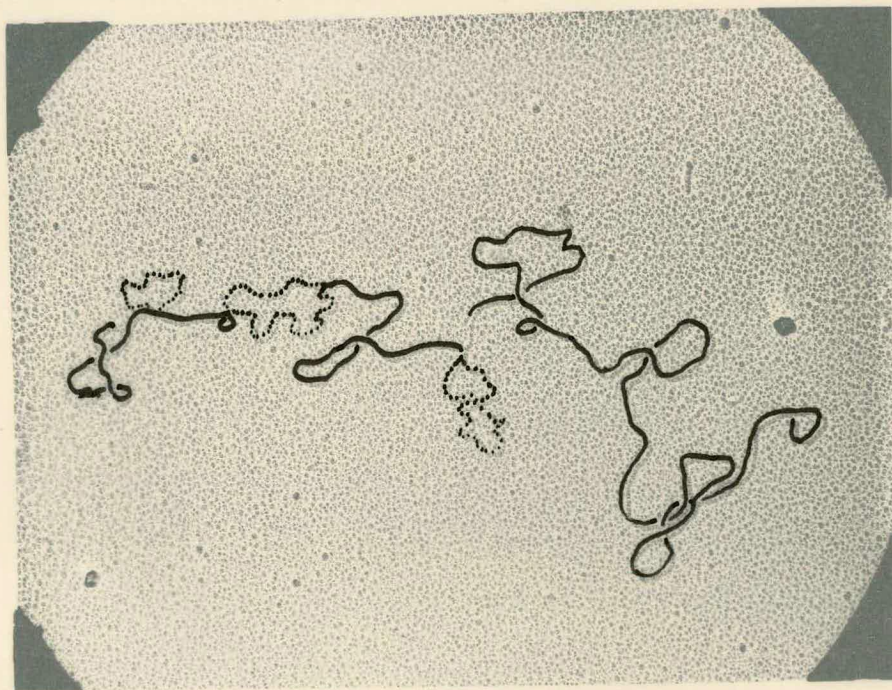
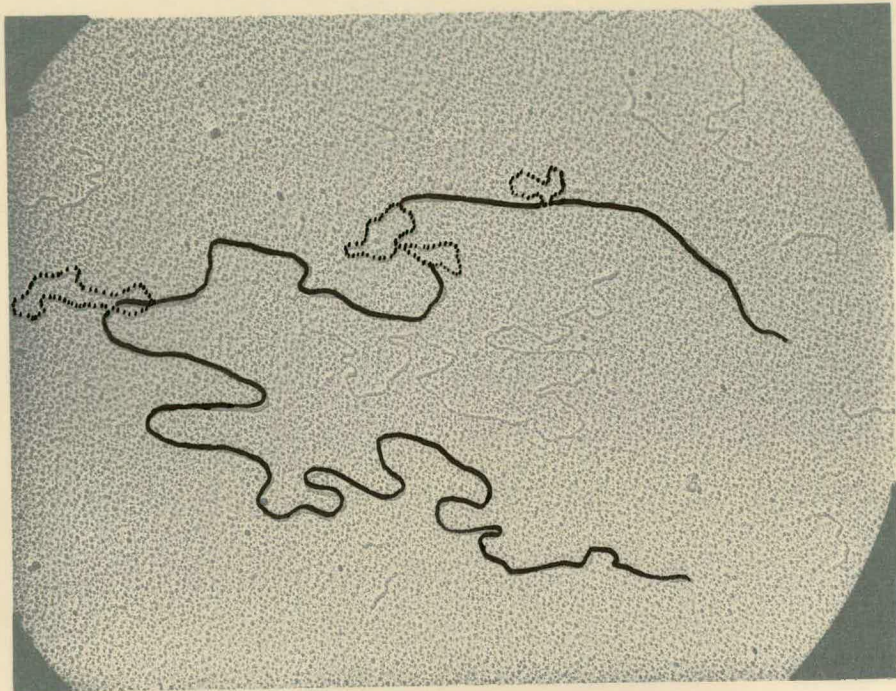


27



28

29



30

most of trpD, (Fig 5), thus complementation demands that the transducing phage carry an intact D gene.

The selection could not be done using a double mutant trpE/trpC host. Trp⁺ plaques arise due to double infections with both types of parental phage. Several trpCDE phages were obtained and four of these were tested for orientation as described earlier. Three of these phage had the fragments from the parental phages in the reverse orientation; both the parental phages have their fragment in the normal orientation. The fourth phage had its trp genes in the normal orientation. All the phages complemented hosts with lesions in trpE, trpD or trpC.

The construction of these phages allows biochemical characterisation of their expression of the genes for the enzyme anthranilate synthetase, without the complication of only having component I of the enzyme present (See Introduction and Methods).

In addition this experiment also confirms that the E and C fragments do end at a R.HindIII target in the trpD gene and that a functional D gene can be re-constituted by ligation of the two separate fragments.

These in vitro constructed trpEDC phages have been designated 1710CDE for the phage carrying trpE, D and C in the normal orientation, and 1017EDC for that of reversed orientation. (The order of the trp gene letter names is the same as that on the vegetative phage chromosome.)

Biochemical Characterization

The in vitro constructed phages 171OCDE and 1017EDC were tested for their ability to direct the synthesis of anthranilate synthetase (ASase), upon infection of the trp BCDE deleted host W3110 trpBE9.

The cells were infected with a multiplicity of 2 phage per cell, in the presence of derepressing (2 $\mu\text{g/ml}$) and repressing (200 $\mu\text{g/ml}$) concentrations of tryptophan, under conditions allowing replication of phage DNA.

The cells were harvested 30 minutes after resuspension in the growth medium following adsorption. The levels of ASase accumulated in this time are given in table 4.

Table 4

Phage	Tryptophan conc. ($\mu\text{g/ml}$)	ASase (Units/mg Protein)
171OCDE	2	0.04
	200	0.01
1017EDC	2	0.004
	200	0.002

Phage 171OCDE, which has its trp genes in the normal orientation, has a ratio of ASase, in the derepressed to repressed state of the operon, of 4 to 1. Phage 1017EDC with reversed orientation has a ratio of 2:1. The residual expression present in high concentrations of tryptophan must represent expression from a phage promoter. In E. coli the repressed level of expression of the chromosomal trp operon is only 1% of that achieved on derepression (Moir 1975).

The ratio of 4 to 1 in the specific activities of ASase under derepressing and repressing conditions, 30 minutes after infection, seen

in phage 1710CDE is typical of expression from the phage promoter P_L with expression from the trp promoter superimposed and subject to repression by tryptophan. This suggests that transcripts initiated at P_L can read through the attachment site and into the inserted trp genes.

The level of ASase obtained seen from phage 1017EDC is only one tenth of that achieved by phage 1710CDE under the same conditions. Curtailing expression from the trp promoter also has a smaller effect on the total level of expression. That there is expression under repressing conditions confirms the earlier evidence for the existence of a promoter initiating transcription rightwards from, or through, the b₂ region. This expression could be due to the promoter responsible for expression of phage 13A (reversed orientation) in the immune state, or it could be the result of transcripts initiated at p_R reaching the b₂ region.

The very low level of expression in phage 1017EDC compared to that seen with phage 1710CDE, (of normal orientation) may be due to interference with transcription from p_{trp} by converging leftward transcription from p_L. Experiments have been conducted to try and observe the effects of repressing expression from p_L in phage 1017EDC. The levels of expression in these experiments, which have to be done in the absence of phage DNA replication, have, to date, been too small to allow any meaningful conclusions to be drawn.

It is possible that in the absence of competitive transcription from p_L expression from the trp promoter in phage 1017EDC might be increased.

Tryptophan Synthetase (TSase)

TSase activity was measured upon infection of the host W3110 trp AC9 tna with phage 19A which carries the trp genes A, B, and C in the normal orientation. Phage DNA replication was permitted as in the experiments to assay ASase activities. After infection at a multiplicity of two, the cells were grown for 30 minutes and TSase activity assayed by conversion of indole to tryptophan. Uninfected cells were used as the standard. Phage 19A accumulated 0.79 Units of TSase per mg of protein in 30 minutes.

TSase specific activity is tenfold higher than the specific activity of ASase when compared under the same conditions (Morse & Yanofsky, 1968). Taking this into consideration the level of TSase measured with phage 19A is still eightfold higher than that seen with phage 171OCDE under conditions of tryptophan repression.

TSase synthesis directed by phage 19A was compared in an immune (lysogenic for λ_{imm}^{21}) and non-immune host. This experiment had to be conducted under conditions preventing phage DNA replication in the non-immune cell. This was achieved by infecting a thymine-requiring derivative of W3110 trpAC9 tna, (obtained by the trimethoprim selection technique), in the absence of thymine and in the presence of 5-fluorouracil. Phages infecting the immune cells (W3110 trpAC9 tna (λ_{imm}^{21})), cannot replicate due to the presence of phage 21 repressor protein. To allow accumulation of measurable quantities of TSase the cells were infected at a multiplicity of nine phage per cell.

Under these conditions phage 19A accumulated 0.185 Units of TSase per mg of protein in the non-immune cell in 30 minutes. Synthesis of

TSase in the immune cell was undetectable over the same period of time. The difference in levels of TSase accumulated in the non-immune cell in this experiment, and over the same time period when the phage were allowed to replicate (approximately four-fold higher) can be accounted for in terms of the number of gene copies of the phage. After thirty minutes of replication there would be about 40 to 50 copies of the phage genome per cell, i.e. four to five times the number infecting the thymine-starved host.

This experiment demonstrates that the expression of the trpABC genes of phage 19A is from a phage promoter which is repressed by the phage repressor protein. Thus, from the known orientation of the phage trp genes this has to be the major leftward promoter p_L. This means that transcripts initiated at p_L can read through the phage attachment site and into the trp genes inserted at R.HindIII site 3.

If the expression of phage 19A is from p_L then the expression of phage 171OCDE under conditions of repression of the trp promoter by high tryptophan will also be from p_L. As mentioned earlier the level of TSase accumulated by phage 19A is eight times higher than the level of ASase accumulated by phage 171OCDE in the same time period. This difference may be due to a combination of a) the presence of the trp attenuator in phage 171OCDE, and b) a terminator in the bacterial chromosome upstream of the trp operon that is able to stop N-activated transcription.

There is no evidence as to whether a transcript initiated at the phage promoter p_L and reading through the trp promoter under conditions of tryptophan repression in a phage-trp operon fusion is able, under the influence of the phage N-protein to read through the trp attenuator site. Franklin, (1974), observed that an in vivo-derived trp-transducing phage,

λtrp9, had a very low level of expression of anthranilate synthetase under conditions of trp repression, although it should have been capable of expressing its incorporated trp genes by readthrough from p_L. She argued that this phenomenon could be explained by the presence of a terminator which in the bacterial chromosome upstream of the trp genes which N-promoted transcription could not pass. The trpE gene fragment carried by the in vitro constructed phages is 11% of the size of the lambda genome; the trpE gene approximately 4%. Thus these phages must have much more bacterial chromosomal DNA upstream of the trp operon than λtrp9 could possibly carry and so if a terminator exists the in vitro phages probably carry it.

The effect observed by Franklin could have been due to a terminator created as a result of the illegitimate recombination event responsible for the genesis of λtrp9. The question of whether the difference in levels of ASase in phage 171OCDE and TSase in phage 19A is due to a terminator or the attenuator or both will have to be answered by comparing, under conditions of trp repression, the expression of TSase from a phage carrying the whole trp operon with an isogenic phage incorporating a deletion of the attenuator site.

Nature does nothing in vain,
And more is vain when less will serve;
For Nature is pleased with simplicity
And affects not the pomp of superfluous causes.

Isaac Newton

DISCUSSION

The E. coli chromosome has a molecular weight of about 3×10^9 Daltons, equal to 4×10^6 base pairs (Klotz & Zimm, 1972). If R.HindIII targets are distributed randomly throughout the DNA molecule a site would be expected once in 4×10^3 base pairs. Thus upon digestion with R.HindIII a thousand fragments would be produced.

The phages characterized in this thesis were collected into "pots" of transfectants; two to four hundred transfectant plaques on a plate were harvested like a phage lysate and those from three to four plates were pooled. Each "pot" therefore contained about one thousand independent transfectant plaques. To find in each "pot" a receptor phage cloning any specific fragment of the E. coli chromosome would require virtually every phage to have acquired an insertion of heterologous DNA. The frequency of a particular type of trp phage was less than one per lysate, but still high; 64% of the "pots" contained trpA transducing phage and 50% trpC transducing phage. The frequency of trpE transducing phage was lower, only 30%. Those trpE phage isolated were predominantly from the first ten "pots" in which more plates were pooled than in later transfections; 1000 to 1200 plaques (3 to 4 plates) in the first ten "pots", only 400-600 (1-2 plates) in the remaining ones. This lower frequency of trpE phage may be correlated with the larger size of the trpE fragment; smaller fragments may be incorporated more easily than larger ones possibly due to their ability to diffuse more rapidly during ligation and thus encounter receptor phage molecules more frequently.

However, by extrapolation, it appears that, as far as smaller fragments are concerned, receptor molecules incorporating heterologous

DNA fragments arise at a frequency greater than 50% of the total recovered. If this is generally true then the construction of special receptor phage chromosomes incorporating selective mechanisms to demand or indicate the acquisition of heterologous DNA fragments may be unnecessary, especially when a selective procedure for the expression of the incorporated genes exists.

There is a striking non-random distribution of orientation of the inserted trp fragments. In the absence of selective pressure it would be expected that fragments would be inserted in either orientation with equal probability. That this is not so suggests that some selective system is operating either at a level affecting the viability of the phages, or due to the trp selection imposed to isolate the phages. Selection for a trp⁺ plaque demands that the phages express their incorporated trp genes at a level to sustain cell growth.

The trpE fragment carries the bacterial trp promoter which should transcribe the E gene normally; the trpC fragment has the constitutive promoter at the promoter distal end of the D gene, expression from which, although only 1% of the derepressed level of expression from p_{trp} allows sufficient trpB and A products to be synthesised for a cell to grow on indole. Thus demanding trp⁺ plaque formation would not be expected to select for orientation of the fragment so as to be expressed from p_L.

The trpA phages isolated do not carry any known bacterial promoter capable of expressing the A gene. Expression of trpA in these phages must therefore be dependent on a phage promoter.

Seven trpA transducing phages were tested and six had their trpA fragment inserted such that it would be expressed from p_L or from the constitutive promoter in the xis gene (Shimada, Weisberg & Gottesman, 1973).

That one was isolated in which the trpA gene was in the reverse orientation suggests that there is leftward transcription of the b₂ region in lambda. The preponderance of trpA transducing phage with trpA in the normal orientation and transcribed from the N-operon may well reflect the efficiency of the leftward promoter p_L.

All the phages characterised in this work, with the exception of phage 19C, are integration proficient. Upon integration the transduced trp genes are physically isolated from the leftward reading N-operon, the trp genes lying just downstream of the host biotin operon. There are three possible explanations as to how the trpA phages of normal orientation can express their trp gene in the lysogenic state.

i) There is a leftward reading, constitutive promoter between the hybrid phage bacterial attachment site and the point of insertion of the trp genes, R.HindIII site 3.

ii) The lysogens screened were tandem di-lysogens in which the expression is due to transcription from the constitutive promoter in the xis gene of the upstream phage reading through the attachment site into trp (Shimada, et al., 1972).

iii) Expression is due to readthrough from the adjacent bioA gene which is transcribed leftwards from the bio promoter towards the prophage (Guha, Saturen & Szybalski, 1971).

If either ii) or iii) are correct then there can be no terminator for transcription between att and R.HindIII site 3.

Of the trpE phages isolated only one has its fragment inserted in the normal orientation. This suggests strongly that, although in either orientation the genes are expressed sufficiently for the phage to be detected as transducing trpE, there may be some selective disadvantage in carrying the fragment in the normal orientation.

Both orientations of the trpE gene fragment show trp expression to be repressible by tryptophan, thus the fragment includes the trp promoter and operator region. (This would be expected from the size of the fragment).

In the normal orientation, the trpE fragment is transcribed on the l-strand of lambda DNA, like a transcript initiated at p_L. The distal end of the trp gene is fused directly into the b₂ region very close to the end of the late gene operon, which is transcribed in the opposite direction, on the r-strand, from p_R. It is therefore possible that transcripts from p_R encounter competition from RNA polymerase molecules transcribing trp in the opposite direction.

The trp promoter on a trpE transducing phage in the b₂ region transcribing leftwards towards the late genes on the antisense DNA strand may interfere with the expression of these genes, possibly reducing the number of viable phage produced upon lysis of the host. This would, in effect, mean that phages with their trp genes inserted in the reversed orientation would have a selective advantage over those of normal orientation. It is also possible that some gene product, coded for upstream of P_{trp}, is overproduced upon transcription from p_L and is detrimental to phage development.

The information derived from these in vitro constructed transducing phages is of great value in assessing the suitability of particular phages for cloning heterologous fragments of DNA. The receptor 540 has the tremendous advantage that transducing derivatives retain an intact attachment site and thus will integrate at the bacterial attachment site forming stable lysogens. This study of trp-transducing phages has shown that such lysogens can express the incorporated genes irrespective of

their orientation on the receptor phage. (This is probably only true so long as the fragment itself does not contain a terminator for transcription). The level of expression achieved by these phages is not very high but it could be high enough to complement mutations in genes coding for proteins required in catalytic rather than stoichiometric quantities in the way that phages 9A or 13A complement a trpA host. Hybrids of lambda with other phages such as P22, P1, P2, and P4 could be used as lysogens to determine the location of a phage gene on a particular fragment created by a restriction endonuclease such as R.HindIII.

It is intended that such a system will be used to derive a genetic linkage map of the satellite phage P4, using λ -P4 hybrids constructed using both the R.EcoRI and R.HindIII enzymes.

The phages analysed in this thesis had the advantage of a selection for their incorporated trp DNA by complementation. In the absence of such a selection system other methods must be used. Such systems have and are being developed.

One is the use of a receptor with a single target for R.EcoRI or R.HindIII in the repressor gene of phage lambda. These phages, upon incorporation of a heterologous fragment, produce clear plaques and can therefore be detected easily in a population of turbid parental plaques. Once isolated, the fragment from these receptors can be translocated in vitro into other receptor phage, eg. of the type described in this thesis, for study of their expression as lysogens, or into a receptor with an endonuclease target in the N-operon so that the phage can be used as a factory molecule to make large quantities of the proteins coded on the fragment.

Integration proficient phage can be lysogenised and the cells grown to high density before induction of the prophage by ultra-violet irradiation or heat shock with a temperature sensitive mutation in the repressor gene. When the phage are defective in late gene expression, Q^- , or lysis defective, S^- , the cells accumulate abnormally high numbers of phage DNA copies, (two to five hundred per cell). If Q^- most of the transcription is confined to the early regions of the genome, ie from p_L and p_R and if cro^- expression from p_L does not turn off but continues unabated. This amplification due to gene dosage and high level expression from p_L allows the synthesis of enormous quantities of proteins from a fragment inserted in the N -operon (Moir, 1975).

The evidence obtained from measurement of the size of the inserted fragments by heteroduplex mapping of the R.HindIII phage 540-trp fusions suggests that when the incorporated fragment is small, there may be selective advantages in the phage acquiring duplications to restore its DNA content nearer to wild type size. In the light of this it may prove wise to relocate such fragments, once isolated, in a receptor that they will restore to near wild type size so reducing the possibility of duplications arising.

Zipkas & Riley (1975) have presented evidence that the E. coli chromosome may have arisen from two successive duplications of a molecule one quarter the size of the present chromosome. Many genes 45 or 22.5 minutes apart bear strong resemblances in their functions. The argF and argI genes both code for an ornithine transcarbamylase and lambda transducing phages derived in vivo carrying the argF or argI genes show regions of homology within these genes when heteroduplexes between them are observed in the electron microscope (Kikuchi & Gorini, 1975).

Recently lambda transducing phages incorporating the E. coli tryptophanase (tna) gene, and the Bacillus licheniformis trp genes have been isolated in vitro in this laboratory by other workers (W. J. Brammar, S. Winton & S. Muir, pers. commun.). The tryptophanase, gene product of E. coli degrades tryptophan to indole, the reverse of the reaction catalyzed by tryptophan synthetase. Tryptophanase can be used by a cell with a complete deletion of the trp operon to convert indole to tryptophan, i.e. it can work backwards to catalyze the same reaction as tryptophan synthetase, and both enzymes use pyridoxal 5-phosphate as a cofactor in this reaction. In the light of this it would seem reasonable to expect that there might be homology between the tryptophanase and tryptophan synthetase genes of E. coli. The in vitro constructed phages carrying tna and trpA provide ideal material for looking for homology in heteroduplexes. If such homology does exist it would give additional support to the theory of Zipkas & Riley.

The isolation of lambda transducing phages carrying the B. licheniformis trp genes will allow comparison of these trp genes with those of E. coli. The lambda-B. licheniformis trp phages were isolated by complementation of E. coli trp auxotrophs in the same way as the phages characterized in this thesis. B. licheniformis is a Gram-positive bacterium and therefore is likely from an evolutionary point of view to be quite divergent from the gram negative bacterium E. coli.

The phages carrying the B. licheniformis trp genes are of two types; they complement for trpABC and D in E. coli, or only for trpBC and D. It has been mentioned earlier that in E. coli the trpA and B gene products work as a complex and neither is active without its partner, therefore for a B. licheniformis trpBCD transducing phage to complement

a trpB mutant E. coli strain the trpB component of B. licheniformis must either be able to work independently of its own A gene protein or else complex with the A protein from E. coli. The same argument can be applied for the trpE gene product which, although able to work independently of trpD protein, is much more efficient when complexed with it. If this is so then it implies that at least the trpA and trpB proteins coded for from the trp operons of the two organisms must be very similar. With such strong similarities between the two systems it is possible that heteroduplexes between lambda-E. coli and lambda-B. licheniformis trp-transducing phages will reveal regions of DNA homology between these two organisms.

Appendix 1

Recombination between R.HindIII targets

The phages described in this thesis almost certainly carry discrete fragments cut from the trp operon of E. coli with the R.HindIII enzyme. These fragments can be joined together in vitro. The E fragment from one phage can be joined to the C fragment of another to reconstitute the trpD gene, the resultant phage transducing trpC, D and E.

Early in the course of the work on these phages some crosses were done to see if adjacent trp fragments would recombine. Phage 17E was crossed with 10C; 10E with 8C; 9A with 10C, and 13A with 8C, all in vivo. (Reference to table 2 will show that the members of each pair carry their transduced fragments in the same orientation). Initially these crosses were done in the trp⁺ host C600.

TrpEDC recombinants were selected on strain trpED102, and therefore have to provide not only E protein but active D as well. Similarly trpABC recombinants were selected on strain W3110 AC9, deleted for all three of these transduced genes (fig 5)*. Both types of recombinant were isolated, the recombination frequency being between 10⁻⁵ to 10⁻⁶. Recombinants were isolated with the transduced genes in the normal orientation (17E x 10C and 10C x 9A), or reversed (10E x 8C and 8C x 13A).

It is possible that these crosses were mediated by homology provided by the trp⁺ host used for the crosses. The crosses were repeated in the host W3110 BE9. This strain is deleted for the genes E through B in the trp operon, thus eliminating possible homology in the regions of crossover, trpB or trpD. Recombinants were detected at the same frequency as before.

All the recombinants were checked that they complemented individually, (i.e. for single genes), or collectively, (i.e. the deletions ED102 and AC9),

*(Fig 5 of Results)

Figures 2A and 2C
mixed and digested
with R. Nihilii

Figure 2B, 2D, 2E
with R. Nihilii

Fig. 2A, 2B
(partial digest)
(control)

Fig. 2A
2C

Fig. 2A
2C

Phage 9ABC digested
with R.HindIII

Phages 9A and 10C
mixed and digested
with R.HindIII

Trp A + C →
(partial digest
product)

trp A →
trp C →

← trp A
← trp C



for the genes they were supposed to carry. Other crosses were done with phages neglected for the most part of the thesis work. Crosses 27C x 8E and 9C x 1E also produced trpCDE recombinants. Three crosses, where the parental phages carried their trp genes in opposing orientations, did not yield the selected recombinants: 10C x 1E; 17A x 9C; 10C x 13A. So this phenomenon is not just a peculiarity of the phages chosen for characterisation.

DNA was isolated from the recombinant 9ABC, progeny of the cross 9A x 10C. This DNA was digested with R.HindIII and the fragments separated on agarose. Plate 31 shows the result of this experiment. In one track is phage 9ABC, in the other a mixture of phages 9A and 10C, digested with R.HindIII. Clearly the recombinant carries two fragments corresponding to the A and C fragments, of the two parents. In addition a larger band can be seen from phage 9ABC which is exactly at the position expected for the two fragments joined together: the result of incomplete digestion.

These experiments strongly suggest the occurrence of legitimate recombination events mediated and confined to the region of homology provided by the R.HindIII targets, six base pairs. Further work is in progress to determine whether the bacterial (Rec) or phage (Red) or some other recombination system is responsible for these events.

It presents the exciting possibility that, given an appropriate selection, two restriction fragments on separate phage could be joined in vivo.

Appendix II

The genetic crosses between Ø80pt190 and the in vitro constructed trp phages, used to determine the orientation of the inserted trp genes, were repeated to compare the frequency of recombination in a recombination proficient host with that in a recA strain. The phages with normal orientation, 9A, 10C, 17E and 19A were crossed with pt190 in the host strains QR47 and QR48 (recA). This yielded the results presented below.

<u>Phage cross</u>	<u>Recombination frequency</u>	
	QR47	QR48
9 <u>A</u> x pt190	4.9×10^{-4}	4.6×10^{-4}
10 <u>C</u> x "	5.4×10^{-4}	1.5×10^{-4}
17 <u>E</u> x "	2.57×10^{-4}	8×10^{-5}
19 <u>A</u> x "	1×10^{-3}	2.5×10^{-4}

Normally in phage x phage crosses the bacterial recombination system makes little contribution to the frequency of recombination since the recBC nuclease is inhibited by the phage gam gene product (Unger & Clark, 1972). These crosses suggest a significant contribution on the part of the host recombination system. McMilin, Stahl & Stahl, (1974), have described the occurrence of what are called chi spots in bacterial chromosomal material carried on transducing phages. Chi stands for Crossover Hotspot Instigator and chi sites are believed to be sequences of DNA which mediate a high frequency of Rec promoted recombinational events.

In the light of this information it seems likely that what has been observed in the above crosses is chi mediated Rec promoted recombination, and that the chi sites occur in the DNA fragments containing the trpC and trpE genes.

The presence of chi sites in lambda-bio transducing phages explains the normal plaque forming ability of these phages. A phage mutant in red and gam usually has a very low burst size, lambda-bio transducing phages do not. It is believed that the chi site(s) in the transduced bio genes stimulate Rec promoted recombination between covalently closed circular replicative intermediates of the phage DNA during development, allowing maturation of a nearly normal number of phages, (Henderson & Weil, 1975). In vivo constructed lambda trp-transducing phages show similar behaviour, and in the light of the evidence presented here it is likely that they too are able to mature due to the presence of chi sites in the incorporated trp operon DNA.

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